In vitro selection and characterisation of iron-efficient potato cell lines

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ABSTRACT

Iron (Fe) is an essential micronutrient involved in life-sustaining biochemical processes including photosynthesis, DNA synthesis and repair, antioxidative defence, hormone synthesis and respiration. Fe bioavailability, however, is a major constraint for crop production due to low Fe solubility resulting from multiple soil and /or biochemical stresses. Iron deficiency-induced chlorosis (IDC) is a widespread nutritional disorder in plants worldwide especially in calcareous soils. To reduce the economic impact of Fe-deficiency stress, it has become necessary to deploy *in vitro* tissue culture as a quick and cost-effective tool for developing stress-tolerant plants. The development of IDC-tolerant (Fe-efficient) plants presents an important means of alleviating IDC. The aim of the present study was to investigate the hypothesis that using *in vitro* selection, it would be possible to exploit somaclonal variation and the inherent ability of plants to evolve adaptive mechanisms under Fe-deficiency stress for the generation of novel Fe-efficient potato plant lines. To achieve this, an *in vitro* selection strategy was designed to generate putative Fe-efficient potato (*Solanum tuberosum* cv 'Iwa') cell lines and plants were regenerated from these cell lines. The Fe-efficient plant lines derived from these cell lines were characterised, using various morphological, biochemical and molecular parameters, regarding their responses to Fe-deficiency under *in vitro* conditions.

A two-step direct selection scheme employing Fe deficiency (0-5 µM) as selective pressure was applied in generating novel Fe-efficient somaclonal variants. Fe-efficient callus cells were selected on half-strength Murashige and Skoog (MS) medium supplemented with 1.78 µM N_6-benzylaminopurine (BA), 3.22 µM α-naphthaleneacetic acid (NAA) and 0-5 µM FeNaEDTA. Shoot buds were regenerated from the calli exhibiting IDC tolerance when cultured on half-strength MS medium supplemented with 6.66 µM BA and 2.89 µM gibberellic acid (GA_3) and 35 plant lines were established. The IDC tolerance of the 35 plant lines was confirmed when they were grown on plant growth regulator-free medium supplemented with 0-5 µM FeNaEDTA. Using IDC scores as the evaluation criteria, 23% of the plant lines (8) could be regarded as Fe-efficient and 77% (27) as Fe-inefficient plant lines.

The compact potato callus cultures produced were used to investigate more closely their responses to a short-term (1 month) and an extended period (3 months) of a Fe deficit or sufficiency in the culture medium. The morphological responses include visual chlorotic symptoms (yellowing), reduced fresh weight and area of growth covered by calli grown on Fe-deficient medium. A Fe deficit in the medium led to decreases in chlorophyll and

carotenoid contents, reduction in activities of peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes accompanied with an increase in lipid peroxidation in calli. Exposure of calli to Fe deficiency enhanced ferric chelate reductase (FCR) activity, induced phenolic production and increased hydrogen peroxide (H_2O_2) generation. Histochemical staining of Fe showed that whereas Fe distribution in cells of calli cultured on Fe-deficient medium was sparse, Fe was widely distributed among actively dividing callus cells cultured on Fe-sufficient medium. The morphological and biochemical responses assessed were pronounced with prolonged exposure to Fe deficiency leading to severe chlorosis and/or death of cells in chlorosis-susceptible calli but chlorosis-tolerant calli cells maintained their greenness and viability. These findings have contributed to a better understanding of Fe nutrition in potato at the cellular level.

The morphological, biochemical and molecular mechanisms conferring differential tolerance to Fe deficiency in two contrasting groups of potato plant lines established in this study were characterised. Fe-inefficient (INF) plant lines and control plants exhibited severe chlorosis when grown on a Fe-limiting medium, but Fe-efficient (EF) lines were tolerant to chlorosis. The EF plant lines were of shorter statures as far as stem height, root length, internodal distances were concerned compared to INF and control plants. Formation of lateral roots and root hairs was enhanced in EF plant lines compared to INF and control plants, suggesting that these might be morphological adaptations in the root of the EF plant lines in response to a deficit in Fe supply. There was a significantly positive correlation between chlorosis score and root length in control plants and INF plant lines, indicating that root length could contribute to IDC susceptibility. Furthermore, stem height was found to have a highly positive relationship with intermodal distance, leaf and root lengths in EF plant lines.

Biochemically, IDC tolerance was linked to increased chlorophyll contents, FCR and POD enzyme activities in the leaves of EF plant lines. The absence of a similar adaptive strategy in leaves of INF lines could underpin their susceptibility to Fe deficiency conditions. FCR and POD activities in the roots of EF plant lines increased greatly while chlorophyll content decreased. The reverse was observed in the roots of INF plant lines, implying that the aforementioned responses contributed to Fe-efficiency at the root level. Increased POD activities in both roots and leaves of EF plant lines suggest that improved capacity to detoxify ROS plays a role to adapt to Fe deficiency stress. Leaf carotenoid content was highly variable among EF and INF plant lines but the carotenoid content decreased in the roots of EF compared to INF plant lines. It would seem that carotenoid content in the roots rather than the leaves might be a more suitable indicator for assessing differential tolerance to IDC. Fe

deficiency resulted in the reduction in phenolic concentration in roots and leaves of IFN and some EF plant lines relative to control plants. However, 40-50% of the EF lines (A1, B2, E1-3) showed higher amounts of phenolics in roots and/or leaves. The findings suggest that IDC tolerance is related to improvement in ferric reductase ability, regulation of chlorophyll biosynthesis and enhancement of the antioxidant potentials. These parameters may serve as predictors for Fe-efficiency trait in plants.

Transcriptional responses of both sets of potato plant lines provide evidence that ferritin (FER3) and the iron-regulated transporter (IRT1) play a role in the acquisition of iron from sources with low bioavailability mainly in the leaves and roots respectively. The A1 chlorosis-tolerant plant line exhibited characteristic Fe-efficiency responses of increased *fer3* and *irt1* transcripts in leaf and root organs. Significantly increased *fer3* expression was detected in the leaves of a higher proportion (62%) of putative-Fe-efficient lines than in roots (37.5%). *IRT1* expression level was 20% significantly greater in roots than in leaves. *IRT1* transcripts in the roots of 50% of the potential EF plant lines (A1, B2, B9, E3) was enhanced in response to Fe deficiency. Some IFN plants had higher values of gene expression compared to EF plants.

In conclusion, a novel set of Fe-efficient and inefficient potato plant lines has been developed which can be useful in further research and breeding programs aiming at selecting IDC tolerant potato genotypes. Taken together, the results showed that 37.5% of the putative EF plant lines (A1, B2, B9) have both the capacity to elicit improved morphological and biochemical characteristics in responses to Fe-deficiency stress as well as to enhance the expression of Fe homeostasis-related genes. The *in vitro* selection approach used in this study can be applied for the selection of Fe-efficiency in other plant species.

DEDICATION

I dedicate this research to God Almighty, my parents (Grace Danka and James Addae), my husband (Dr. Louis Boamponsem) and children (Baruch and Theresa).

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ABBREVIATIONS

AHA	Arabidopsis H^+-pump ATPase
APX	ascorbate peroxidase
BA	N_6-benzylaminopurine
bHLH	basic helix-loop-helix
bp	nucleotide base pair
BPDS	bathophenantroline disulfonic disodium salt
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary DNA
CIM	callus induction media
cm	centimetre
Ct	cycle threshold
DAB	diaminobenzidine
DEPC	diethyl pyrocarbonate
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleoside triphosphate
EDTA	ethylenediamine tetra acetic acid
FAO	Food and Agriculture Organization
F-C	Folin-Ciocalteu
Fe	iron
FeNaEDTA	iron sodium ethylenediaminotetraacetate
FRC	ferric chelate reductase
FRO	ferric reductase oxidase
Fw	fresh weight
g	gram
$gmol^{-1}$	gram per mole
GA_3	gibberellic acid
H_2O	water
HCl	hydrochloric acid
IDC	iron deficiency-induced chlorosis
IRT1	iron-regulated transporter

L	litre
MES	2-(4-morpholino) ethanesulfonic acid)
mg	milligram
min	minute
ml	millilitre
mm	millimetre
Mn	manganese
MS	Murashige and Skoog
NA	nicotianamine
Na_2CO_3	sodium carbonate
Na_2EDTA	disodium ethylenediamine tetraacetic acid
NAA	α-naphthaleneacetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NO	nitric oxide
NRAMP	natural resistance associated macrophage protein
^oC	degree Celsius
%	percentage
PGR	plant growth regulators
PMSF	phenylmethylsulfonylfluoride
POD	peroxidase
PPB	Perls Prussian blue
ppm	parts per million
PTC	plant tissue culture
PVP-40	polyvinylpolypyrrolidone
qPCR	real-time polymerase chain reaction
RDA	recommended daily allowance
RM	Regeneration medium
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcription
s	second

S.E.	standard error
SE	standard error (of the mean)
SM	selection medium
SV	somaclonal variation
TAE	tris-acetate-EDTA
µg	microgram
µl	microliter
µm	micrometre
µM	micromoles per litre
UV	ultraviolet
Zn	zinc

CHAPTER ONE

General introduction

1.0. Importance of studying the biological significance of iron

Iron (Fe) is an indispensable micronutrient involved in life-sustaining biochemical processes in organisms (Thomine and Vert, 2013; Ravet et al., 2009). Iron deficiency is a nutritional disorder affecting billions of people worldwide. It is also prevalent in plants due to multiple soil and / or biochemical stresses that limit Fe bioavailability. Fe deficiency can therefore have a significant negative impact on the economic development of a nation. There has been limited success with the use of iron-fortified foods and supplements to tackle iron malnutrition because these interventions are expensive and not readily accessible to the rural poor.

Biofortification, a recent food-based approach to produce micronutrient-rich crops, holds great potential in combating iron malnutrition. Most cultural and traditional corrective applications to solve Fe deficiency in plants are labour-intensive, costly and can be detrimental to the environment. *In vitro* selection of plants capable of growing in Fe-deficient environments (Fe-efficient plants) using plant tissue culture (PTC) techniques is a cost-effective and alternative tool to avert Fe-deficiency problems. There has been extensive research regarding the use of PTC in the screening and selection of valuable agronomic traits in plants. However, little attention has been paid to the selection of Fe-efficient plants using potato callus cultures. *In vitro* selection can complement existing approaches and conventional field selection methods of acquiring Fe-deficiency resistant plants.

This study seeks to employ plant tissue culture to examine the responses of potato plants to Fe supply and to select for Fe-efficient cell lines. The cell lines are then characterised based on morphological, biochemical and molecular parameters. This chapter presents a review of the functions of iron in humans and plants, mechanism of iron uptake in plants and *in vitro* selection of desirable traits. A detailed description of the objectives of the present study is presented at the end of this Chapter.

1.1 Iron in living systems

Iron (Fe) is a common metal found in the Earth's crust and water bodies. It is a transition metal that can change its oxidation state both in complexes with organic molecules

(octahedral complexes with different ligands) and in hydrated free states, $[Fe\ (H_2O)_6]^{2+}$ and $[Fe\ (H_2O)_6]^{3+}$ (Darbani et al., 2013; Ha¨nsch and Mendel, 2009; Hall and Guerinot, 2006). Due to this property, iron plays a key role in redox reactions involving the gain or loss of an electron. This makes it an essential nutrient for the electron transport reactions of the photosynthesis, respiration and energy transfer chains (Ha¨nsch and Mendel, 2009; Roschzttardtz et al., 2013). Iron is crucial for living cells since it is a key component of many important macromolecules associated with metabolism. Fe is required for DNA synthesis and serves as a cofactor for a variety of enzymes (Thomine and Vert, 2013; Perron and Brumaghim, 2009; Ravet et al., 2009).

1.1.1 Iron and human health

The unstable nature of Fe with its ability to donate or accept electrons makes it versatile and capable of performing various functions within cells. Functions of iron in humans include the following; antibodies and collagen synthesis, conversion of carotene to vitamin A, detoxification of drugs in the liver, fat removal from the blood and catalyse electron and oxygen transport processes (Long and Shannon, 1983; Woods et al., 1990). Iron is used in the bone marrow to make new red blood cells and is a key component of haemoglobin (oxygen carrier in red blood cells).

The persistent lack of micronutrients including iron in diet is regarded as one of the most critical global challenges (Poletti and Sautter, 2005; Carvalho and Vasconcelos, 2013**;** Saltzman et al., 2013). Awareness on chronic micronutrient malnutrition, also known as "Hidden Hunger", has increased in the 21st century (Poletti and Sautter, 2005; Pfeiffer and McClafferty, 2007; Stein, 2010; Haynes et al., 2012**)**. Some of the underlying causes of iron deficiency anaemia (IDA) include poverty, low dietary intake of iron-rich foods and insufficient agricultural development. Iron deficiency impairs the quality of life and lowers national productivity levels as well as reduces agricultural output. IDA is the most prevalent and serious nutritional disorder worldwide with an estimated 30% (over 3 billion people) of the world's population affected (WHO, 2011; Hindt and Guerinot, 2012; Lucca et al., 2002; Nestel et al., 2006; Hirschi, 2009). It is the only nutrient deficiency with a significant presence in industrial nations (WHO, 2011). The most vulnerable populations with the highest prevalence of IDA are infants, adolescents, pregnant women and people in famine-affected regions. IDA adversely affects approximately 50% of pregnant women and 40% of preschool children mostly in developing countries (WHO, 2011).

Over the years, efforts to tackle iron malnutrition centred on providing iron supplements and chemically fortified foods. Lack of economic will and adequate infrastructure can negatively affect the supply of supplements and fortified foods especially in developing nations. There has been limited success with iron supplementation (by way of administering pharmaceutical preparations) because it is expensive, usually has low compliance due to the unpleasant side effects of medicinal iron and the notion that artificial drugs may be harmful to health (McClafferty and Russell, 2002; Lucca et al., 2002). In contrast, biofortification, a recent food-based and cost-effective intervention approach to produce micronutrient-enhanced crops, holds great potential as one of the most effective novel tools in combating micronutrient malnutrition worldwide.

1.1.2 Iron nutrition in plants

In plants, iron plays a critical role in chlorophyll formation, photosynthesis, respiration, hormone synthesis (gibberellic acid, ethylene, and jasmonic acid), nitrogen fixation, osmoprotection, pathogen defence, production and scavenging of reactive oxygen species, sulphate assimilation, DNA synthesis and repair (Vasconcelos and Grusak, 2014; Darbani et al., 2013; Zamboni et al., 2012; Hindt and Guerinot, 2012; Ha¨nsch and Mendel, 2009; Kerkeb and Connolly, 2006). Iron content in plant-based foods is largely reliant on the bioavailability of soil iron. The capacity of plants to utilise iron is affected by factors such as the ionic Fe species (Fe^{2+} or Fe^{3+}) in the growth medium, macro- and micronutrient imbalance, and bicarbonate ion concentration. In most parts of the world, dietary iron is mainly obtained from plant-based foods but a considerable number of such foods are poor sources of iron because they contain low or insufficient levels of iron (Kerkeb and Connolly, 2006).

Optimum plant growth is supported and maintained by an estimated 10^{-9} to 10^{-4}M soil iron (Hell and Stephan, 2003; Guerinot and Yi, 1994; Zuo and Zhang, 2011). Fe can be a toxic element depending on plant growth conditions. Most plants have, however, evolved mechanisms that enable them to adapt to Fe-poor or Fe-overload environments. Low bioavailability of Fe in soils is a major agricultural problem because it results in Fe-deficiency in crops plants. The deficiency of Fe^{2+} can restrict chlorophyll synthesis, induce interveinal chlorosis (expressed as yellowing in leaves ending with necrosis and fall of leaves in the more serious situations), reduce crop quality and yields as well as decrease the nutritional value of edible plant parts (Bert et al., 2013; García-Mina et al., 2013;

Vasconcelos and Grusak, 2014; Abadia et al., 2011; Hindt and Guerinot, 2012). Chlorosis is a condition that generally refers to a lack of chlorophyll and is associated with a number of abnormalities in plants. In potato, marked decreases in tuber quality and yield, biomass, chlorophyll concentrations and alterations in enzyme activities (peroxidase, catalase and acid phosphatase) were observed under Fe deficit stress (Chatterjee et al., 2006). Susceptibility to Fe chlorosis is based on a plant's response to Fe deficiency stress and this is genetically regulated (Simko et al., 2008). The potato gene that confers the phenotype with chlorotic and malformed leaves is denoted *cml* and other chlorosis mutations such as *virescens* (v), yellow margin, green (g), and light green (lg) are reported in potato (Simko et al., 2008). The authors suggest that these mutations are conferred and mostly controlled by single recessive genes.

1.1.3 Factors affecting iron availability in soils and plants

Iron is the fourth most abundant element in the Earth's crust but the third-most limiting nutrient for plant growth principally because it forms complexes that are not readily amenable to uptake by plant roots (Guerinot and Yi, 1994; Zuo and Zhang, 2011). In most aerobic environments and / or soils of high pH (7 - 9) the concentration of soluble or bioavailable iron is several orders of magnitude lower, ranging between ~10^{-17} and 10^{-10} M (Hell and Stephan, 2003; Kerkeb and Connolly, 2006; Zuo and Zhang, 2011) because iron forms water-insoluble hydroxides and oxides, and / or Fe carbonates–bicarbonates. Fe(III) undergoes polymerisation, hydrolysation [$Fe(OH)^3$] and precipitates with inorganic anions at increasing pH values (Hell and Stephan, 2003). This makes Fe acquisition by plants a very challenging problem. Fe deficiency in plants can result from multiple soil and /or biochemical stresses and is not solely limited to the availability of Fe in the environment. Factors linked with Fe deficiency include soil salinity, low temperature, high pH, carbonates, high moisture content, low water drainage, soil bulk density, high nitrate concentration, interactions of Fe with other soil minerals, landscape position across and within the same farmland (Vasconcelos and Grusak, 2014; Hansen et al., 2006). Such factors hinder Fe absorption or impair Fe use in metabolic processes.

Fe deficiency is prevalent in plants growing on calcareous and alkaline soils. The pH of most calcareous soils is within the range of 7.5 to 8.5. Such soils contain over 15% free calcium carbonate ($CaCO_3$) and occur naturally in arid and semi-arid regions due to relatively little leaching (Zuo and Zhang, 2011). The carbonates have relatively high reactivity, solubility and alkaline character. Calcareous soils usually have low organic matter content

and are low in nutrients. Fe, zinc (Zn), manganese (Mn) and Cu deficiencies are common in soils that have a high $CaCO_3$ due to reduced solubility at alkaline pH values. Calcareous soils may contain high levels of total Fe, but in forms unavailable to plants. Reduced nutrient availability in calcareous soils lead to lime-induced chlorosis and stunted growth in many crops (Bert et al., 2013; Naik et al., 1990; Abadia et al., 2011). Leaf Fe concentration is not essentially related to degree of chlorosis since Fe concentrations in chlorotic plants can be higher or same as non-chlorotic plants – a phenomenon termed 'iron chlorosis paradox' (Bavaresco et al. 1999; Romheld, 2000). Therefore, chlorosis on calcareous soils is not always attributable to Fe deficiency and is referred to as lime-induced Fe chlorosis.

It is proposed that one third (30%) of the world's cultivated soils should be classified as calcareous and deficient in Fe (Kim and Guerinot, 2007; Hansen et al., 2006; Barton and Abadía, 2006; Guerinot and Yi, 1994). Semiarid soils cover 1% of New Zealand and occur in the inland basins of Otago and Southern Canterbury (Hewitt, 2010). They have lime and salts accumulated in the lower subsoils. The chemical properties of these soils include; low iron, low organic matter, cation exchange capacity, aluminium oxide contents and they are weakly buffered (Hewitt, 2010). Calcareous cliffs can be found on Miocene sediments along the coast of Eastern Wairarapa and parts of Western Waikato in the Northern Island. In the South Island, coastal calcareous cliffs occur in Punakaiki, in South Marlborough (near Flaxbourne Stream), Kaikoura coast as well as in Motunau Beach and Napenape of North Canterbury (Kennedy and Dickson, 2007).

1.2 Iron acquisition in plants

An essential criterion for iron acquisition by plants is the iron status of the soil. When there is sufficient iron for plant growth, plant roots reduce Fe(III)-chelates and transport Fe(II) through the plasma membrane (Legay et al., 2012; Hindt and Guerinot, 2012). To cope with low Fe solubility, plants have evolved strategies for Fe acquisition – Strategy I and Strategy II as proposed by Romheld and Marschner (1986). Strategy I plants employ a reduction-based strategy while strategy II plants use the chelation-based mechanism for the acquisition of iron. Graminaceous monocots such as maize (*Zea mays*), barley (*Hordeum vulgare*) and rice (*Oryza sativa*) apply a chelation-based strategy (Hansch and Mendel, 2009; Kerkeb and Connolly, 2006; Ivanov et al., 2012). Strategy II plants synthesise Fe chelators (phytosiderophores) which can bind Fe (III) with high affinity. The Fe (III) phytosiderophore complexes are then transported through a specific transport system (Rodríguez-Celma et al.,

2013; Grotz and Guerinot, 2006; Kerkeb and Connolly, 2006; Zamboni et al., 2012). Eudicots and non graminaceous monocots employ a reduction-based Fe acquisition mechanism. Potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), *Arabidopsis thaliana*, rapeseed (*Brassica napus*), pea (*Pisum sativum*) and fruit trees employ a reduction-based strategy (Legay et al., 2012; Ivanov et al., 2012). Iron uptake in potato follows the strategy I mechanism which involves the release of protons to make iron more soluble in the rhizosphere, leading to reduction of Fe (III) to Fe (II) and the transport of Fe (II) from the roots to other parts of the plant (Legay et al. 2012). Iron uptake, complexation, distribution and storage are highly controlled so that cells can obtain a sufficient supply of iron for metabolic processes.

The current knowledge gap in mechanisms leading to iron acquisition and nutrition in potato appears to be a major impediment in devising approaches to improve growth of crops in marginal soils and for biofortification. It is therefore crucial that an understanding of the machinery and mechanisms controlling plant iron utilisation and homeostasis is developed. This is beneficial for human health and nutrition since iron deficiency is a serious nutritional problem worldwide. This section provides comprehensive information on iron acquisition in strategy I plants.

1.2.1 The Strategy I mechanism for Fe acquisition - Proton release

Plants exposed to iron-deficient conditions first acidify the soil to increase iron solubility and subsequently reduce Fe (III)-chelates through a specific root reductase reaction. With the reduction strategy, all higher plants except graminaceous monocots release protons through root plasma membrane H^+-ATPases belonging to the Arabidopsis H^+-ATPase (AHA) family (Santi and Schmidt, 2009; Hindt and Guerinot, 2012). The release of protons induced in the roots of Fe-deficient plants serve to acidify the rhizosphere by decreasing the soil pH; a unit drop in pH results in a thousand-fold rise in Fe solubility (Grotz and Guerinot, 2006; Guerinot and Yi, 1994). Acidification of the rhizosphere helps to drive more iron into solution and cause an increase in Fe (III) solubility in the soil. AHA7 has been identified to be up-regulated in response to Fe-deficiency (Yi and Guerinot, 1996). In cucumber, expression of CsHA1, an iron-regulated proton ATPase, is induced in Fe-deficient roots (Santi et al., 2005).

1.2.2 Fe (III) Reduction

Following rhizosphere acidification is the reduction of Fe^{3+} to the more soluble Fe^{2+} by a ferric chelate reductase (FCR) and subsequent uptake of Fe^{2+} into plant root cell by a Fe(II) transporter (Grotz and Guerinot, 2006; Rodríguez-Celma et al., 2013; Zamboni et al., 2012). FCR accepts cytosolic electrons, transfer the electrons to Fe^{3+} through heme molecules across the membrane and then converts the Fe^{3+} to Fe^{2+}; reduction of Fe (III) to Fe (II) increases the solubility of iron for plant uptake (Kerkeb and Connolly, 2006; Ghandilyan et al., 2006; Legay et al., 2012). This phenomenon seems to support the suggestion that reduction of iron is the rate-limiting step in iron uptake by Strategy I plants (Connolly et al., 2003; Grusak et al., 1990; Jeong and Connolly, 2009).

FCR is a member of the ferric reductase oxidase (FRO) family of metalloreductases in plants and belongs to a superfamily of flavocytochromes. The role of FRO in iron uptake and homeostasis has been discovered, characterised and documented across various plant species including Arabidopsis (FRO2: Yi and Guerinot, 1996; Robinson et al., 1999), pea (PsFRO1: Waters et al., 2002), tomato (LeFRO1: Li et al., 2004), soybean (FRO2: Vasconcelos et al., 2006), potato (FRO1: Legay et al., 2012). Robinson, et al. (1999) were the first to identify FRO2 as the enzyme required for the plasma membrane ferric chelate reductase activity in the roots of Arabidopsis plants exposed to iron deficiency conditions. FCR is proposed to play a vital role in Strategy I response because the Arabidopsis *FRO2* loss-of-function mutant, *frd1*, developed severe chlorosis when grown on iron-deficient medium. Also *frd1* showed no inducible root FCR activity and was defective in iron uptake when iron is supplied as a Fe^{3+} chelate (Yi and Guerinot, 1996).

A number of FROs have been identified and predicted to be localised to organellar membranes. This lends credence to the presumption that some FROs may have functions other than reduction of iron. The expression of FRO5 was detected to be induced under iron deficiency (Mukherjee et al., 2006) but recent studies by Bernal et al. (2012) have revealed and established that FRO4 and FRO5 act to reduce copper at the root surface. Arabidopsis FRO6, FRO7and FRO8 genes are expressed in the green shoot tissues of plants (Mukherjee et al., 2006; Grotz and Guerinot, 2006) and the expression of FRO3 in the roots and shoots is activated by iron limitation (Mukherjee et al., 2006; Waters et al., 2002). FRO6 expression was induced by light (Jeong and Connolly, 2009) and its promoter was found to contain multiple light responsive elements (LREs) using a GUS (β-glucuronidase) report gene technique (Feng et al., 2006).

1.2.3 Fe (II) uptake into roots

Reduced iron (Fe^{2+}) is transported across the root plasma membrane by Fe(II) transporters. The iron-regulated transporter (IRT1) protein, a member of the ZIP (Zn-Fe-regulated transporter like proteins) family of metal transporters, serves as the main machinery for the entry of Fe (II) into root cells. IRT1 is a divalent cation transporter with a high affinity for Fe^{2+} (Vert et al., 2002; Henriques et al., 2002) but also transports other metals such as Zn, Mn, cadmium (Cd), and cobalt, Co (Eide et al., 1996; Vert et al., 2002; Hindt and Guerinot, 2012). At low metal concentrations, iron may be preferentially taken up over other metals since IRT1 has strong affinity for Fe^{2+}.

IRT1 gene was initially detected in Arabidopsis by the use of a yeast functional complementation approach (Eide et al., 1996). IRT1 is localised in the plasma membrane (Vert et al., 2002; Eide et al., 1996; Hindt and Guerinot, 2012) and its expression is activated in the epidermis of roots by iron deficiency (Vert et al., 2002; Hindt and Guerinot, 2012; Eide et al., 1996; Kerkeb and Connolly, 2006). An IRT1 knockout mutant with loss of IRT1 activity (*irt1-1*) depicted decreased growth, reduced iron accumulation, modified chloroplast morphology and severe chlorotic symptoms but such effects were restored after more iron was supplied to the growth medium (Henriques et al., 2002; Vert et al., 2002; Hindt and Guerinot, 2012; Kerkeb and Connolly, 2006). The *IRT1* gene is expressed in the basal part of flowers, suggesting its role in Fe uptake in aerial tissues as well as in roots (Vert et al., 2002). IRT2 can transport Fe and Zn similar to IRT1 and is expressed in the epidermal cells of Fe-deficient roots but overexpression of *irt2* in *irt1* mutants could not restore the phenotypes observed in *irt1* mutants (Vert et al., 2002; Grotz and Guerinot, 2006). This seems to suggest that IRT1 and IRT2 have different functions. IRT1 homologues have been identified in other Strategy I plants such as tomato (Eckhardt et al., 2001) and pea (Cohen et al., 2004).

Specific regulatory mechanisms enable plants to control IRT1 levels to ensure optimal absorption of iron and to prevent iron toxicity caused by excess iron. It has been reported that there is a local (through the root) and systemic (shoot-derived signal) regulation of *IRT1* expression (Vert et al., 2003; Hall and Guerinot, 2006). Overexpression of *irt1* in *A. thaliana* showed post-transcriptional control of IRT1 (Connolly et al., 2002). Accumulation and subcellular localisation of IRT1 is important for effective iron uptake, plant growth and development (Barberon et al., 2011). The authors showed that IRT1 is ubiquitinated *in vivo* and that monoubiquitination of IRT1 controls its trafficking from the plasma membrane to the vacuole where it undergoes degradation. Analysis of constitutive expression of *irt1* in

transgenic plants demonstrated that movement of IRT1 to its degradation site occurred independently of the state of iron nutrition (Barberon et al., 2011).

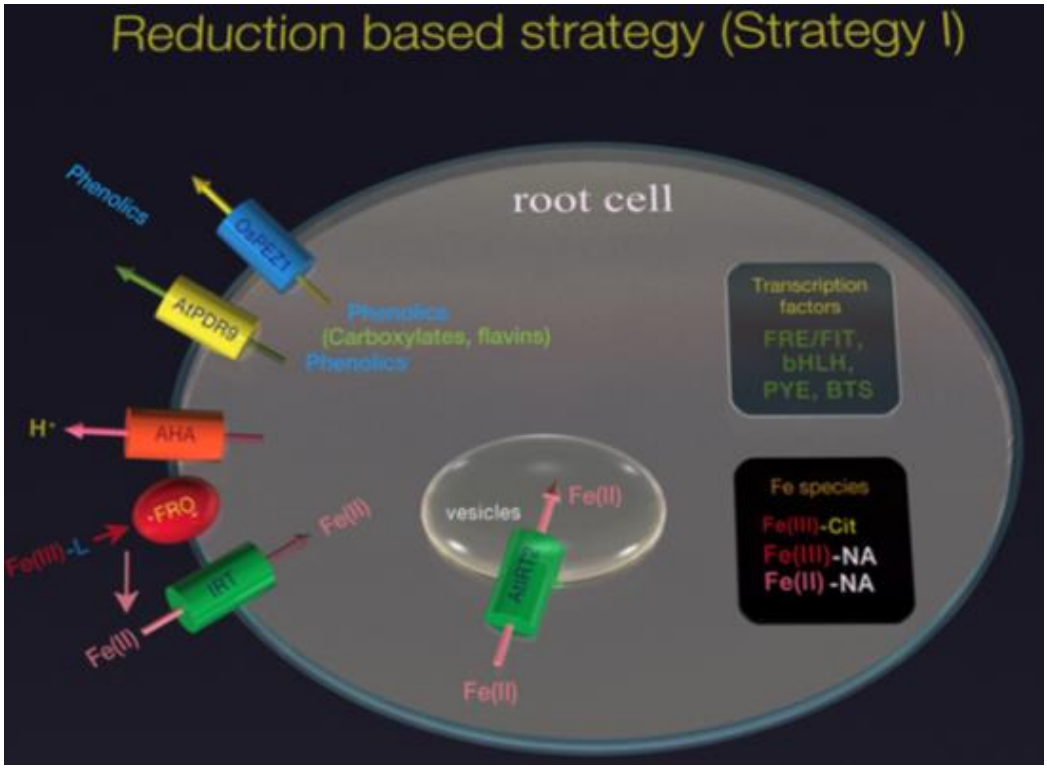


Figure 1. Strategy I reduction-based iron uptake mechanisms and the membrane transport processes involved under Fe-deficiency. The system is also regulated by transcription factors (FRE/FIT, bHLHs, POPEYE: PYE, BRUTUS*:* BTS). FRO2: ferric chelate reductase, IRT1: iron regulated transporter. An Arabidopsis H^+-ATPase (AHA) excretes protons to the apoplast, pleiotropic drug resistance 9 (AtPDR9) and the phenolic efflux zero1 (OsPEZ1) transporters excrete phenolics. Taken from Abadía et al. (2011).

1.3 Long-distance Fe transport

After plant roots have acquired iron, the iron is distributed to other tissues and organs. Iron is transported from the root epidermis to the central vascular cylinder for xylem loading and translocated to the aerial sections of the plant (Rogers, 2006; Briat et al., 2007). The vascular cylinder consists of the xylem and the phloem. The xylem transports water and solutes from the root to the shoot whereas the phloem transports metabolites from the shoot to the root. Transport of iron to various plant organs begins with release of free Fe^{2+}within the apoplast (Kerkeb and Connolly, 2006). In order to by-pass the Casparian band (layer of suberin-coated endodermal cells), solutes enter the vascular cylinder through the symplastic pathway. For

entry into xylem vessels Fe is effluxed into the apoplastic space from the symplast. In the xylem (with pH of 5.5–6), iron is present as Fe (III) complexed with citrate (Hell and Stephan, 2003).

1.3.1 Shoot iron supply

The ferric reductase defective3 (FRD3) transmembrane protein, a member of the multidrug and toxin efflux (MATE) family of small molecule exporters, is suggested to play a role in shoot iron localisation (Rogers and Guerinot, 2002; Green and Rogers, 2004). *FRD3* gene expression was identified mainly in root tissue (Rogers and Guerinot, 2002), an indication that FRD3 protein could be involved in xylem loading. Analysis of FRD3-FLAG constructs and FRD3-green fluorescent protein (FRD3-GFP) fusion proteins showed that the protein is expressed in the pericycle and cells neighbouring the xylem and phloem (Green and Rogers, 2004). A map-based approach was used to clone the *frd3* gene. The results were verified by complementation of the FRD3 mutant phenotypes using genomic DNA with the putative *frd3* gene insert and observing the occurrence of sequence modifications in mutant alleles (Rogers and Guerinot, 2002; Rogers, 2006). Available immunofluorescence and localisation data of FRD3 protein show that it is localised in the plasma membrane (Green and Rogers, 2004; Rogers, 2006).

1.3.2 Xylem transport

Rogers and Guerinot (2002) proposed that FRD3 exports into the xylem as a low molecular-weight iron-chelate complex essential for unloading Fe from the xylem in the shoot. According to the authors limited iron in root tissue activated *frd3* transcript levels to almost double its iron-sufficient expression. They observed that *frd3* mutant leaves had lower levels of the iron storage protein, ferritin, which served as an indirect biomarker of iron levels in cells. It is assumed that FRD3 transports citrate-Fe complex into the xylem and molecular analysis of the *Arabidopsis thaliana* mutant, *frd3,* has given proof of the role of citrate in iron translocation (Durrett et al., 2007; Kim and Guerinot, 2007; Rogers, 2006).

Growth of the *frd3* mutant under highly bioavailable Fe conditions led to an increased build-up of iron in its leaves more than its roots which over accumulated a range of metals together with iron (Rogers and Guerinot, 2002). The shoots and leaves of *frd3* mutant plants grown on soil with low iron level contained 10% less iron than wild type (Lahner et al., 2003). The *frd3* mutant is chlorotic and constitutively expresses Strategy I iron uptake

responses. These characteristics seem to be due to defective localisation of iron in root and the shoot of *frd3* mutant plants coupled with alterations in iron homeostasis (Rogers, 2006; Rogers and Guerinot, 2002). The *frd3* mutant had high amounts of iron in the vascular cylinder of the root but seems to have difficulty in transporting iron through and out of the xylem into the shoot symplast and the chloroplasts (Green and Rogers, 2004; Rogers, 2006). Perls staining revealed high levels of ferritin mainly localised in the vascular cylinder of *frd3* mutant roots (Green and Rogers, 2004). The *frd3* mutants are incapable of efficiently transporting iron from the root to the shoot in a form used by leaf cells (Rogers, 2006). An iron replete *frd3* shoot sent signals to the roots to cause the suppression of iron uptake responses. The grafting studies revealed that the FRD3 function was required in the root to present a wild-type phenotype. This result was in agreement with the localised expression of FRD3 in the root (Rogers and Guerinot, 2002; Rogers, 2006). Addition of citrate to growth medium repaired the chlorotic appearance of *frd3* plants (Kim and Guerinot, 2007).

1.3.3 Phloem transport

Movement of iron from older leaves to younger leaves occurs by means of phloem transport (Kim and Guerinot, 2007; Briat et al., 2007). In leaf cells, Fe^{2+} is distributed to other cellular compartments to function in chlorophyll synthesis and act as a co-factor for enzymes while the excess is stored in ferritin for future use (Kerkeb and Connolly, 2006). Due to the limited solubility and highly reactive nature of Fe, Fe ions have to be chelated after uptake into the cell. To maintain high solubility in the phloem sap (pH 7.2-8.5), Fe has to be attached to chelators. Nicotianamine (NA), a non-proteinogenic amino acid, chelates and transports micronutrient metal ions in plants (Briat et al., 2007; von Wiren et al., 1999; Curie et al., 2009; Legay et al., 2012). NA can bind strongly to Fe^{2+} or Fe^{3+} while it has also been found to be involved in the long-distance transport of copper as well as in Zn homeostasis (Weber et al., 2004; Pich and Scholz, 1996). Based on the ability of NA to form stable complexes with Fe and in the presence of low amounts of Fe^{2+} in the phloem sap, NA has been suggested to play a role in Fe transport in the phloem (von Wiren et al., 1999; Kim and Guerinot, 2007; Kerkeb and Connolly, 2006). NA is essential for iron homeostasis and in the internal transport of Fe (II) and Fe (III) in Strategy I plants like potato (Legay et al. 2012). Data obtained by Legay et al. (2012) denotes that the expression of nicotianamine synthase is influenced by iron deficiency in the plant.

Nicotianamine synthase (NAS) enzyme catalyses the synthesis of NA from three molecules of S-adenosyl-l-methionine (Douchkov et al., 2002; Briat et al., 2007). The chloronerva mutant was detected as a spontaneous mutation in Bonner Beste tomato and constitutively expresses iron uptake responses as well as interveinal chlorosis (Ling et al., 1999). The iron-deficiency phenotype observed in the chloronerva mutant is due to the inability of the plant to synthesise NA (Higuchi et al., 1999). The chloronerva gene was detected to have sequences highly similar to the NAS-like sequences of barley using map-based cloning suggesting that the chloronerva gene encodes nicotianamine synthase (Ling et al., 1999; Higuchi et al., 1999; Hall and Guerinot, 2006). The iron uptake responses in the NA-less tomato mutant were reversed when exogenous nicotianamine was incorporated in growth medium (Bereczky et al., 2003; Hall and Guerinot, 2006). In Arabidopsis, overexpression of the *NAS* gene (AtNAS1) resulted in improved growth and high levels of iron under Fe-deficient condition (Hindt and Guerinot, 2012).

The characteristic high reactivity and limited solubility of iron requires that Fe ions be chelated after uptake into the cell to prevent its participation in the Fenton reaction. At high internal levels of iron, an increased amount of NA was measured in the vacuoles of tomato and pea plants whereas NA was detected in the cytosol under optimal and sub-optimal Fe conditions (Higuchi, et al., 1999; Pich et al., 2001). The increase is perhaps to protect the cells against oxidative damage since the Fe-NA complex does not partake in the Fenton reaction. At high iron concentrations, NA was found to accumulate in the vacuole (Pich et al., 2001) as was detected in iron over-accumulating mutants, an indication that NA may be associated with vacuolar iron sequestration (Hindt and Guerinot, 2012).

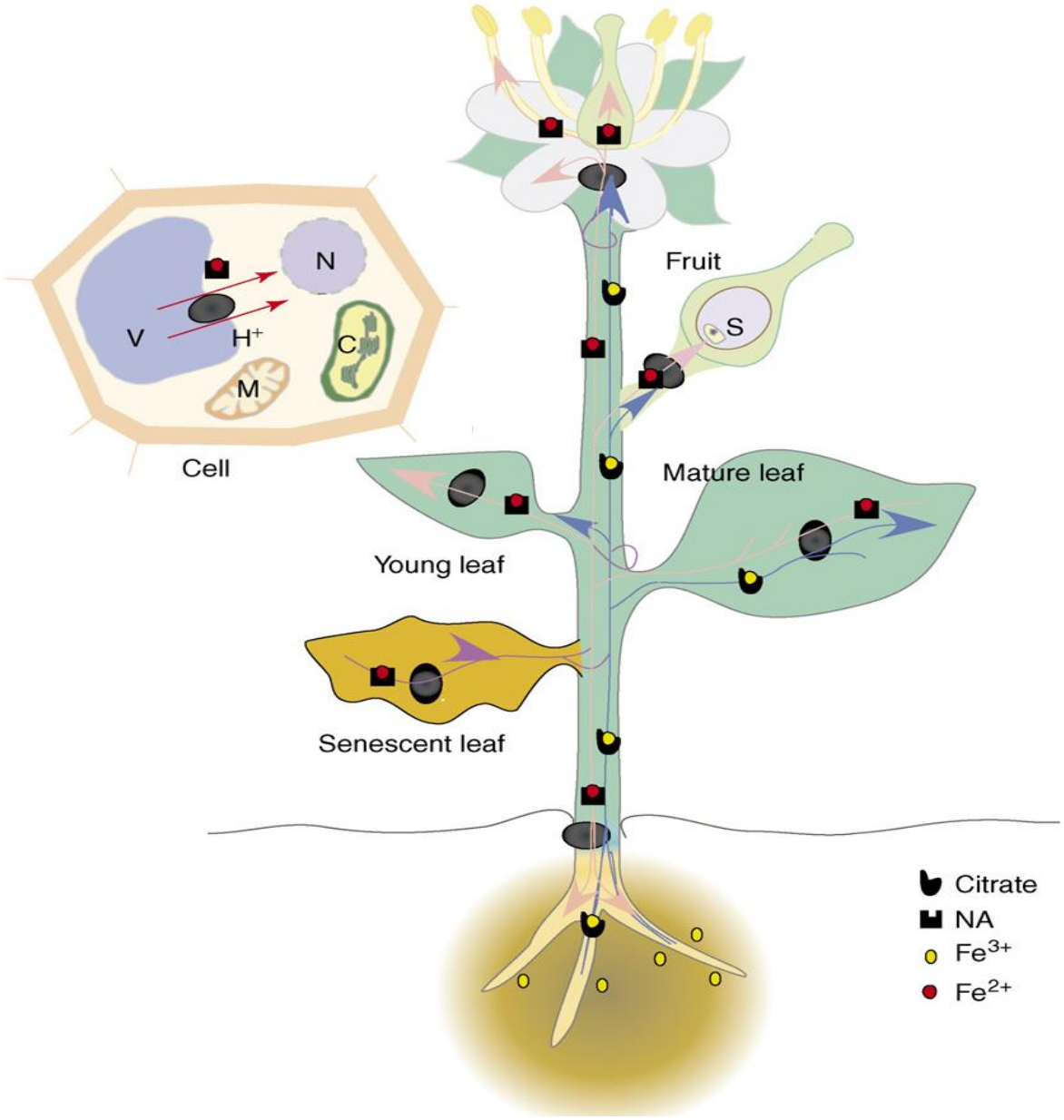


Figure 2. Long-distance circulation of iron chelates within a flowering plant. Iron-citrate (blue arrows) and iron-nicotianamine (pink arrows) circulating in xylem vessels and in phloem vessels respectively. Purple lines signify communications between the vessels. The putative locations of Yellow Stripe-Like transporters along the way (dark ovals) are indicated. C, chloroplast; M, mitochondria; N, nucleus; S, developing seed; V, vacuole. Taken from Briat et al. (2007).

1.4 Intercellular and intracellular iron distribution

Intercellular and intracellular transport of Fe is made possible by the natural resistance associated macrophage protein (NRAMP) family of transporters. NRAMPs are a class of

integral membrane proteins that can transport a variety of metals and have been found to be present in plants, fungi, bacteria and animals (Hindt and Guerinot, 2012; Grotz and Guerinot, 2006). Three of the seven members of the NRAMP family occurring in Arabidopsis have been associated with Fe transport (Grotz and Guerinot, 2006). Natural resistance against microbial pathogens3 in *Arabidopsis thaliana* (AtNRAMP3) and AtNRAMP4 have been shown to function as intracellular Fe transporters mostly involved in vacuolar mobilisation of Fe. AtNRAMP3 is localised in the vacuolar membranes of roots and leaves (Thomine et al., 2003; Hindt and Guerinot, 2012). NRAMP3 remobilises vacuolar Fe into cytosol to cause down-regulation of Fe uptake genes (Grotz and Guerinot, 2006; Kim and Guerinot, 2007). Down-regulation of *irt1* and *fro2* transcripts resulting from overexpression of AtNRAMP3 is suggested to cause an increase in Fe levels in the cytosol as a signal that deactivates the Fe deficiency-induced genes, *fro2* and *irt1*(Thomine et al., 2003). Legay et al. (2012) studied NRAMP3 expression in potato under iron deficiency and were of the view that NRAMP3 possibly released stored iron in the cytosol to compensate for the lack of iron. It is assumed that AtNRAMP1 prevents Fe toxicity by transporting excess Fe into the plastids (because of its plastid targeting sequence) implying that AtNRAMP1 functions to distribute Fe and enable plant growth in a highly concentrated (toxic) Fe environment (Grotz and Guerinot, 2006).

The vacuolar iron transporter 1(VIT1) expressed in the vasculature of roots and shoots, has been implicated in the transport of Fe into the vacuole while NRAMP3 and NRAMP4 are considered to be responsible for the pumping of Fe out of the vacuole into the cytosol (Grotz and Guerinot, 2006). *Nramp* genes have also been identified in tomato, rice and soybean (Bereczky et al., 2003; Kerkeb and Connolly, 2006).

1.4.1 Fe storage: intracellular iron reserves in plants

Iron is predominantly stored in the vacuoles where its uptake is facilitated by VIT1 as discovered in *Arabidopsis thaliana* (Kim et al., 2006; Darbani et al., 2013). Using iron imaging techniques, considerable amounts of Fe were detected in the vacuoles of endodermal cells of mature Arabidopsis and elevated concentrations in the nucleolus of Arabidopsis leaves and pea embryo (Kim et al., 2006; Thomine and Vert, 2013). Storage and release of iron from vacuoles is dependent on the vacuolar pH and phytase activity. High amounts of organic acid and low pH are ideal conditions for Fe deposition in the vacuoles (Briat et al., 2006). An increase in vacuolar pH due to the upregulation of metal transporters restricts or

lowers iron release from phytate in order for iron to be stored. For iron to be released from the vacuole, vacuolar pH is reduced (Darbani et al., 2013).

Ferritin, a plastidial iron storage protein functions both in iron homeostasis and detoxification (Briat et al., 2010; Ihemere et al., 2012). Iron needed for the biosynthesis of Fe-containing proteins can be stored briefly in ferritins (Briat et al., 2006). Due to the highly reactive nature of free iron, binding of iron to macromolecules (ferritin) ensures its availability in a safe (unreactive) form in the chloroplasts (where iron demand is high) and in other compartments; the ferritin protein complex can hold up to 4500 iron atoms (Kerkeb and Connolly, 2006). Chloroplasts are regarded as high iron accumulation sites in plant cells because of the presence of a complete electron transfer chain containing 22 iron atoms that are used as cofactors (Roschzttardtz et al., 2011a, 2013). Ferritin occurs primarily in green plastids (chloroplasts where photosynthesis is active) but can also be found in the mitochondria and non-green plastids, like etioplasts or amyloplasts (Darbani et al., 2013; Briat et al., 2006, 2010; Kerkeb and Connolly, 2006). The chloroplasts and mitochondria are important iron sites within plant cells: mesophyll cells in chloroplast contains about 70–90% of cellular iron (Thomine and Vert, 2013; Briat et al., 2006). A number of key metabolic processes that occur in the chloroplast and the mitochondrion require iron as a cofactor. With photosynthesis (a reaction requiring Fe) being a major function of leaves, the leaf serves as a major site for Fe accumulation in plants (Thomine and Vert, 2013). With the exception of some fungi, ferritin is present in virtually all living organisms (Darbani et al., 2013). Ferritin expression is transcriptionally and post-transcriptionally regulated (Kerkeb and Connolly, 2006). In plants, there are two ferritin isoforms, H1 (26.5 kD) and H2 (28 kD), which are synthesised by post-translational modification processes and presumably have different functions (Ravet et al., 2009; Darbani et al., 2013). Cross communication between compartments regulates allocation of intracellular iron: mutations leading to enhanced Fe sequestration in vacuoles result in down regulation of ferritin levels in the plastid (Ravet et al., 2009).

1.5 Iron detection techniques employed in plant studies

Insight into the formation of cellular Fe stores, its localisation and distribution in cells has been made possible through the advancement of imaging techniques adapted for plant studies. Imaging approaches, histochemistry, micro particle-induced x-ray emission (PIXE) and synchrotron radiation micro X-ray fluorescence (SXRF) have been used to examine and

identify different intracellular iron pools in plant cells. The analysis of iron in Arabidopsis seed using x-ray fluorescence (XRF) tomography provided useful data on the specific localisation and accumulation of Fe and the role of the vacuolar membrane transporter, VITI in Fe homeostasis (Kim et al, 2006). A loss-of-function mutation in VITI resulted in an alteration in the pattern of Fe distribution, leading to a diffuse localisation in the sub-epidermal region of the radicle and the cotyledons. Lanquar et al. (2005) employed inelastic scattered electron microscopy and energy-dispersive X-ray (EDX) microanalysis to measure and localise metals in Arabidopsis during seed germination.

Development of imaging techniques has been key to obtaining new insights into the localisation of Fe in the seeds (Roschzttardtz et al., 2009; Cvitanich et al., 2010) and in the nucleus (Roschzttardtz et al., 2011a). Roschzttardtz et al. (2009) applied histochemical staining using ferrocyanide and diaminobenzidine (Perls/DAB) to detect Fe in Arabidopsis embryo. A sensitivity and resolution equivalent to μXRF was achieved. It was found that Fe is principally stored in the endodermis cell layer, within the vacuoles of dry seeds, and that the Fe is remobilised from the vacuoles during germination. In earlier research, Green and Rogers (2004) and Stacey et al. (2008) employed Perls stain to detect Fe in Arabidopsis plant roots and embryos, respectively. Using the Perls method, Cvitanich et al. (2010) revealed the distribution of Fe in *Phaseolus* seeds and identified iron in the cytoplasm of epidermal cells, cells near the epidermis, and cells surrounding the provascular tissue. The analysis of iron localisation within cells using a combination of histochemistry, PIXE and XRF has led to the discovery of measurable amounts of iron in the nucleus (Roschzttardtz et al., 2011a). Perls/DAB staining together with the DNA fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI), was employed by Roschzttardtz et al. (2011a) to label the nucleus. The level of iron detected in the nucleus was higher than in the plastids or vacuoles which are normally considered as iron-rich organelles.

1.5.1 Mechanism of Perls staining for histochemical detection of iron

The Perls staining procedure is specific for Fe and is a comparatively simple, quick and low cost nonetheless powerful technique that has been widely used in plant research (Roschzttardtz et al., 2009, 2011b and 2013; Cvitanich et al., 2010; Brumbarova and Ivanov, 2014). Staining of Fe can be intensified to obtain images of high resolution. The resolution of the Perls images can be enhanced based on the redox activity of Prussian blue to detect Fe location at the cellular or subcellular levels in plant samples. Perls/DAB staining is a

powerful method to pinpoint Fe-containing compartments in plant cells. Prussian blue breaks down H_2O_2 linked to 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and this activates the formation of a dark-brown coloration due to the oxidative polymerization of DAB (Roschzttardtz et al., 2009). Two reactions occur when both Fe^{2+} and Fe^{3+} are present in a sample. In these reactions, it shown that the addition of H_2O_2 reacts with potassium ferrocyanide and that the intensification process is Perls stain dependent (Roschzttardtz et al., 2009). Intensification of the Perls stain with DAB enhances the sensitivity and definition of Fe detection in plants. Perls staining has been applied at the subcellular, organ and whole plant levels, either with DAB intensification (Reyt et al., 2015; Ivanov et al., 2014; Roschzttardtz et al., 2011b, 2013) or without (Green and Rogers, 2004; Stacey et al., 2008; Cvitanich et al., 2010).

1.5.2 Histochemical detection revealing Fe localisation and distribution in plants

Roschzttardtz et al. (2013) showed that in *Arabidopsis thaliana* roots, iron is localised primarily in the central cylinder while there was a low level of Fe in the epidermis and cortex regions. By investigating the location of iron during its translocation from the roots to the shoot through the vascular system, Fe was observed to amass in the stele (chiefly in the cell walls) with intense staining around the pericycle cells and the xylem tracheary elements. In the stele, the apoplast has been proposed to be a reservoir of Fe and to function in the buffering of Fe during the process of transport toward the aerial parts (Roschzttardtz et al., 2013). Previous studies on Perls staining of root sections of *frd3* mutant plants demonstrated that Fe was concentrated in the central cylinder in Arabidopsis mutant genotypes (Green and Rogers, 2004). They found that there was a build-up of Prussian blue precipitates in the vascular tissues of *frd3* mutant roots but that was largely reduced in the wild-type tissues. Similarly, Roschzttardtz et al. (2013) observed that the intensity of staining for Fe in the *frd3* mutant was significantly enhanced compared to wild type. Fe staining observed within the endodermal cells of *frd3* roots was linked to the possible role of the Casparian strip in limiting apoplastic Fe in the inner layers of the root. The mutant plants were impaired in citrate loading in the xylem. Owing to the findings that iron acquisition in pollen, embryo and leaf relies on FRD3, Roschzttardtz et al. (2011b) put forward that FRD3 mediated-citrate release in the apoplastic space signify an essential route by which efficient iron nutrition occurs between neighbouring tissues that do not have symplastic connections. Results on root Fe distribution (Roschzttardtz et al., 2013) substantiate earlier reports on the role of FRD3 in

facilitating Fe transport between symplastically disconnected tissues (Roschzttardtz et al., 2011b).

To investigate whether Fe was more concentrated around the leaf veins, Roschzttardtz et al. (2013) stained sections of the vascular system and the surrounding cells. A majority of the staining within leaf cells was located in chloroplasts confirming previous data which indicated that 70% of leaf Fe was found in chloroplasts (Shikanai et al., 2003; Roschzttardtz et al., 2013). Perls/DAB staining was useful in the identification of Fe-ferritin in the chloroplast of mesophyll cells and in xylem parenchyma cells. In ferritin mutants, the extracellular compartment of leaf cells was found to contain considerable amounts of iron suggesting an effect of the loss of ferritin on iron homeostasis (Roschzttardtz et al., 2013). Roschzttardtz et al. (2011a) observed intense nuclear staining of Fe in *A. thaliana* and tomato leaves.

Iron in flowers is deemed to be essential for plants because sterility is common in mutants defective in iron homeostasis. Several genes that encode iron acquisition components are expressed in flowers (Stacey et al., 2002; Vert et al., 2002; Connolly et al., 2003). Roschzttardtz et al. (2013) observed large pools of Fe in the anthers and pollen grains of flowers. Within the anther, iron accumulated mostly in cell layers, including the epidermis and endothecium. Iron was detected in the exine layer of the pollen coat and in the cytoplasm at three stages of pollen development. In mature pollen, iron was randomly distributed in the cytosol. The structures with high levels of Fe stains in pollen grains nearby the vegetative nucleus were suggested to correspond to amyloplasts or mitochondria. The authors detected that Fe was highly accumulated in starch-rich structures (amyloplasts) in pollen grains but the nucleolus was not stained with Perls/DAB.

In mature *Arabidopsis* embryos, the vacuoles were detected to be a major iron storage site (Roschzttardtz et al. 2009). Similar to Arabidopsis and tomato leaves, pea embryo was found to have measurable amounts of iron in the nucleus (Roschzttardtz et al., 2011a). The presence of Fe pools in the nucleus, particularly in the nucleolus of pea embryos illustrates that the vacuolar storage of iron is not a general characteristic of seeds (Roschzttardtz et al., 2011a). They reported that the amount of iron in the nucleolus was the highest among the intracellular compartments. Furthermore, the authors suggested that iron in the nucleolus might be directly bound to rRNA. Later research (Roschzttardtz et al., 2013) established that the presence of Fe in the nucleolus could be a common feature of plants and may be required for the catalytic activity of the nucleolus.

1.6 Iron homeostasis - regulation of Fe deficiency responses in Strategy I plants

Iron uptake, complexation, distribution and storage are highly controlled so that cells obtain sufficient supply of iron for metabolic processes to achieve proper growth and development (Darbani et al., 2013; Ivanov et al., 2012; Kim and Guerinot, 2007; Roschzttardtz et al., 2013). Plants have evolved a conserved set of coordinated responses that ensure the maximal uptake and utilisation of iron so that iron levels within cells are balanced (Darbani et al., 2013; Kim and Guerinot, 2007). Responses to iron deficiency in strategy I plants include hormonal signalling, root morphological modifications, secretion of root exudates, alterations in iron uptake-related gene expression and oxidative stress responses.

1.6.1 Role of transcription factors and the 'ferrome'

Initial insight into the regulation of iron deficiency responses in Strategy I plants was obtained from experiments using the tomato mutant, *fer*, which was unable to induce Fe deficiency responses upon iron limitation and was observed to be severely chlorotic (Brown and Ambler, 1974). The *fer* gene was discovered to encode a basic helix–loop–helix (bHLH) transcription factor that regulates iron-deficiency response in tomato (Ling et al., 2002; Bauer et al., 2007). FER is a transcription factor proposed to function in low iron signalling pathway in tomato (Walker and Connolly, 2008). In Arabidopsis, a FER-like-iron-deficiency induced transcription factor, FIT, exhibits a similar expression pattern to FER (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). FIT transcript and protein are induced and accumulated under Fe-deficient conditions (Hindt and Guerinot, 2012). When FIT1, the Arabidopsis characterised protein with high resemblance to FER (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005), was expressed in the tomato *fer* mutant, the plants regained the ability for induction of Fe deficiency responses (Yuan et al., 2005). Expression of Arabidopsis *fit* in tomato complemented the *fer* mutation confirming that it is a functional ortholog of *fer* in Arabidopsis (Yuan et al., 2005). Similar to *irt1* plants, severe symptoms of chlorosis observed in *fer* and *fit1* mutants were reversed when plants were supplied with Fe; *fit* plants only survived and grew when supplied with iron (Vert et al., 2002).

FER mainly occurs in root epidermal cells (Ling et al., 2002; Grotz and Guerinot, 2006) and its expression is enhanced in response to iron deficiency and is required for regulation of the Strategy I Fe-deficiency responses (Ivanov et al., 2012). FIT1, like FER is root-specific: it has been found that the FIT transcription factor coordinates iron deficiency

induced responses in root epidermis of Arabidopsis plants (Walker and Connolly, 2008). Sivitz et al. (2012) reported that under low iron conditions, FIT binds to and up-regulates *fro2, irt1* and other iron-regulated genes in root epidermal cells. FIT seems to have multiple functions: induction of transcription of *fro2* and *irt1* and the prevention of turnover of IRT1 protein when iron is low (Thomine and Vert, 2013). In Arabidopsis, genes that encode Fe(III)-chelate reductase (AtFRO2) and Fe(II) transporter (AtIRT1) are co-regulated by FIT under Fe starvation (Hindt and Guerinot, 2012; Colangelo and Guerinot, 2004).

Using expression profiling experiments some members of the basic helix-loop-helix (bHLH) family (BHLH38, BHLH39, BHLH100 and BHLH101) were found to be involved in iron deficiency response (Yuan et al., 2008). The authors suggested that FIT and BHLH38/BHLH39 induce the expression of *fro2, irt1* and the Strategy I iron-uptake system since FIT with either BHLH38 or BHLH39 were co-expressed in yeast cells under IRT1 and FRO2 promoters. FIT is hypothesised to regulate the expression of *irt1and fro2* by possibly forming a heterodimer (Yuan et al., 2005; Colangelo and Guerinot, 2004; Jakoby et al., 2004) with product(s) of some bHLH (bHLH38, bHLH39, bHLH100, and bHLH101) genes (Hindt and Guerinot, 2012; Yuan et al., 2005,2008). *FRO2* and *IRT1* expression were observed to be elevated in plants that overexpressed both FIT and bHLH38 or bHLH39. These plants accumulated Fe to a greater extent than the wild type (Yuan et al., 2008). When iron is in limited supply, the *fit* gene is activated to up-regulate iron-responsive genes by forming a complex with bHLH proteins (Ivanov et al., 2012). This is because the bHLH genes are also activated by Fe-deficiency (Yuan et al., 2005, 2008).

FIT is controlled transcriptionally and translationally based on the iron nutrition of the plant and other iron uptake signals (Yuan et al., 2005; Thomine and Vert, 2013). FER protein levels seem to be regulated by Fe availability but accumulation of FER transcripts is not dependent on Fe availability indicating post-transcriptional regulation. Upon expression of *fer* under CaMV 35S promoter and examination of FER protein levels in 35S-FER plants, FER mRNA was detected in roots irrespective of Fe status of the plant but FER protein was up-regulated at low Fe levels (Brumbarova and Bauer, 2005; Grotz and Guerinot, 2006). Post-transcriptional regulation of FIT through its breakdown by proteosomes ensures that FIT is rendered dysfunctional and therefore prevented from inducing gene expression when Fe supply is increased in the cells (Hindt and Guerinot, 2012).

The term “ferrome” is used to describe the set of genes involved in metal homeostasis particularly those associated with response to iron deficiency (Zamboni et al., 2012). Transcriptome analysis has shown that 44 genes are over 1.5-fold upregulated while 24 genes

were down-regulated under Fe deficiency induced conditions (Rawat et al., 2004; Kim and Guerinot, 2007). In Strategy I plants, lack of Fe increases the expression of the transcription factors *AtFIT* (*SlFER* in tomato), *AtbHLH38, AtbHLH39*; ferric reductase *AtFRO2* (*SlFRO1* in tomato, *CsFRO1* in cucumber), the iron transporter *AtIRT1* (*SlIRT1* in tomato, *CsIRT1* in cucumber), the H^+-ATPase, *AtAHA7* (*CsHA1* in cucumber), the nicotianamine synthase *AtNAS1* and *AtNAS2*, and the citrate efflux transporter *AtFRD3* (Buckhout et al., 2009; Walker and Connolly, 2008; García-Mina et al., 2013).

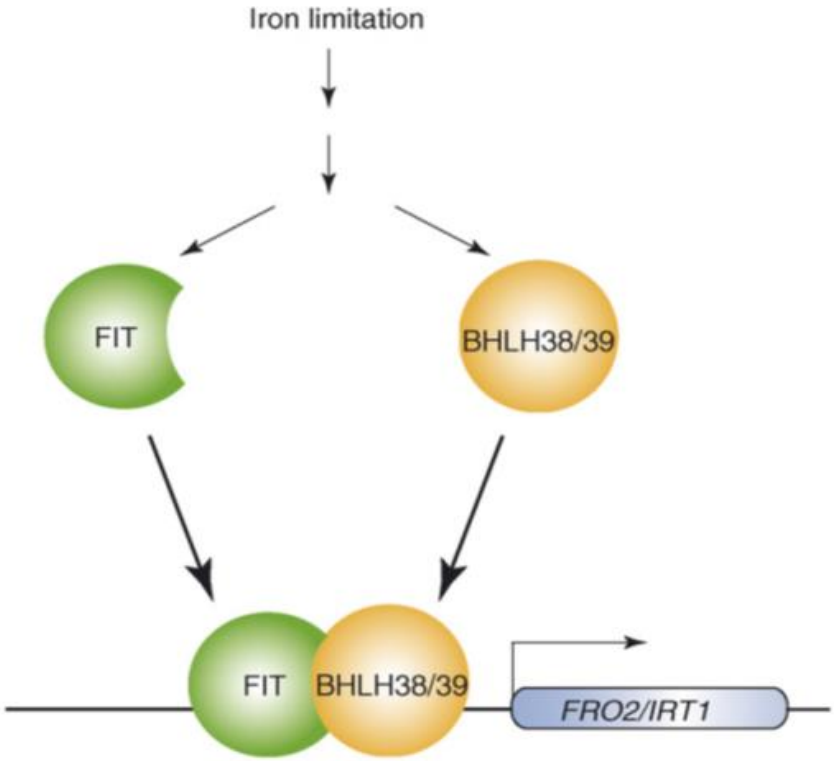


Figure 3. Schematic representation for the induction of *FRO2* and *IRT1* by FIT and BHLH38/BHLH39. FIT, BHLH38, and BHLH39 expression is elevated under iron limitation. FIT heterodimerises with either BHLH38 or BHLH39 to induce transcription of FRO2 and IRT1. Taken from Walker and Connolly (2008).

1.6.2 Hormonal signalling mechanisms in response to iron deficiency

Auxin, ethylene, and NO have been implicated as positive regulators of the *FIT, FRO2,* and *IRT1* genes in an Fe-deficient environment in order to facilitate Fe acquisition (Hindt and Guerinot, 2012; Lucena et al., 2006). Auxin plays a role in the physiological and root morphological responses to Fe deficiency (Schmidt et al., 2000; Chen et al., 2010; Hindt and

Guerinot, 2012). *FIT* and *FRO2* expression was discovered to be enhanced in an auxin over-producing mutant, *yucca,* compared to that wild type (Chen et al., 2010). Although auxin inhibition reduced the expression of *fit* and *fro2,* supply of exogenous auxin induced transcription of these genes (Hindt and Guerinot, 2012).

Auxin and ethylene are produced when Fe is low. They have been found to participate in the regulation the Fe-deficiency response in Strategy I plants by positively influencing FIT or FER expression (Waters et al., 2007; Hindt and Guerinot, 2012, Lucena et al., 2006). Exposure of Fe-deficient tomato and Arabidopsis plants to an ethylene precursor resulted in increased expression of *FER* or *FIT, FRO* and *IRT* genes (Hindt and Guerinot, 2012). On the other hand, a down-regulation of ethylene genes occurred upon treatment of Fe-starved plants with ethylene inhibitors. The ethylene transcription factors, Ethylene Insensitive3-Like1 (EIL1) and Ethylene Insensitive3 (EIN3), interact with FIT, and are important for expression of FIT downstream target genes (Lingam et al., 2011). FIT breakdown by proteosomes is reduced when FIT interacts with EIN3/EIL1 thereby increasing Fe acquisition gene expression (Lingam et al., 2011). This model proposes an increase in Fe acquisition by stabilising FIT instead of a direct role of FIT in the induction of *irt1* and *fro2* expression (Hindt and Guerinot, 2012).

Nitric oxide (NO) functions in hormonal signalling in plants and is presumed to be an iron-deficiency signalling molecule (Hindt and Guerinot, 2012). Incorporation of nitric oxide averted chlorosis in maize mutants defective in Fe uptake (Graziano and Lamattina, 2005) while in tomato, Fe deficiency stimulates NO accumulation (Graziano and Lamattina, 2007; Hindt and Guerinot, 2012). Addition of NO positively regulates Fe utilisation in Fe-deficient tomato roots by activating *FRO1, IRT1,* and *FER* expression but application of NO inhibitors causes a down-regulation of these same genes by decreasing FIT protein levels and its activity (Graziano and Lamattina, 2007). NO seems to stabilise FIT protein most probably by hindering proteosomal degradation of FIT (Hindt and Guerinot, 2012).

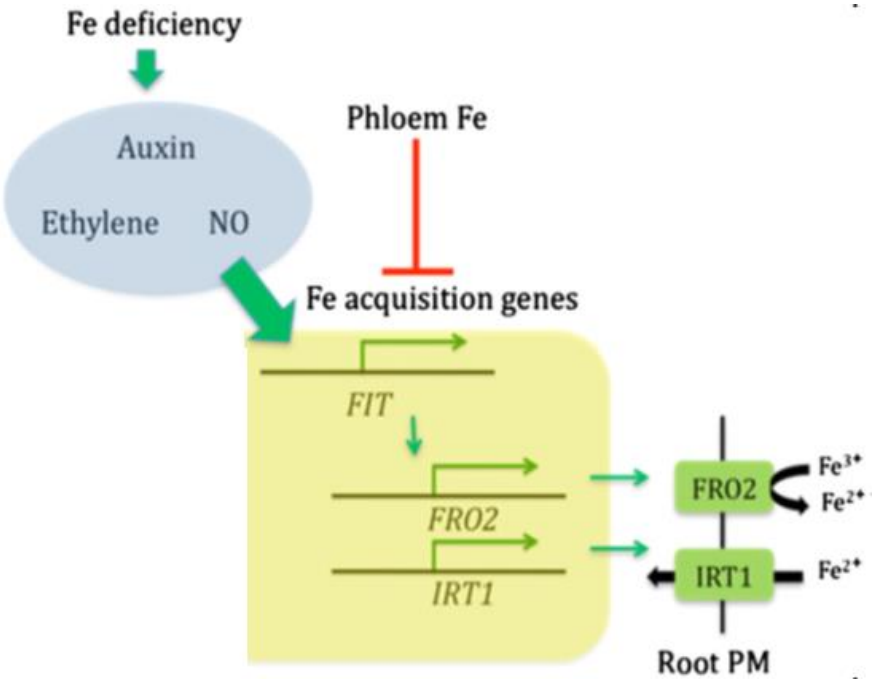


Figure 4. Influence of hormonal signalling on regulation of the Strategy I Fe deficiency response. Auxin, ethylene, and NO are positive regulators of the FIT, FRO2 and IRT1 genes for Fe acquisition. Fe from the phloem can serve as a negative regulator of Fe deficiency gene expression. Model taken from Hindt and Guerinot (2012).

There is usually an interplay among auxin, ethylene, and NO in plants. Increase in auxin seems to activate NO which subsequently induces Fe acquisition (Chen et al., 2010; Hindt and Guerinot, 2012). Chen et al. (2010) indicated that auxin functions upstream of NO in the expression of some Fe-acquisition genes. Auxin and NO were detected to enhance the expression of *AtFIT* and *AtFRO2* in Arabidopsis plants grown in a medium without Fe but in high Fe concentrations, these genes were not expressed (Chen et al., 2010). Ethylene and NO are suggested to jointly regulate genes associated with Fe-deficiency response (Hindt and Guerinot, 2012; Garcia et al., 2011). Previous studies demonstrated that NO formation enhanced ethylene synthesis in roots and that the reverse was also true (Garcia et al., 2011; Hindt and Guerinot, 2012). Stimulation of ethylene and NO in roots due to Fe deficiency increased an activating signal which enhanced FIT, AtbHLH38 and AtbHLH38 expression and ferric reductase activity (Garcia et al., 2011; Chen et al., 2010).

1.6.3 Morphological modifications of the root in response to iron deficiency

Some Strategy I plants respond to low iron concentrations by formation of sub-apical root hairs and transfer cells (Schmidt, 1999; Schmidt et al., 2000; Barton and Abadía, 2006; García-Mina et al., 2013). The roots are laterally elongated to reach out for available iron

(Thomine and Vert, 2013). Iron limitation activates the development of root hairs at regions originally occupied by non-hair cells, a process dependent on auxin and the auxin influx transporter (Schmidt et al., 2000; Thomine and Vert, 2013). This leads to formation of bifurcated root hairs with two tips. Extensin, a structural protein mainly formed in root hair cells, has been observed to be involved in root hair formation (Bucher et al., 2002; Zamboni et al., 2012). Proliferation of lateral roots and root hairs were attributed to the expression of extensin proteins since a strong positive modulation of three genes (*LeExt1, LeDif10, LeDif54*) that encode extensins was detected in Fe-starved tomato plants (Zamboni et al., 2012).

Another morphological adaptation of the root to low Fe conditions is the enhancement of root surface area resulting from swelling of root tips together with increased synthesis of lateral roots and branching root hairs (Schmidt, 1999; Hindt and Guerinot, 2012). In Arabidopsis, root surface area was enlarged by increasing the number and length of root hairs. In tomato however, it was found that the creation of epidermal transfer cells with large invaginations increased root epidermal surface area (Schikora and Schmidt, 2001). The increased root surface area facilitates iron reduction and transport of iron.

1.6.4 Secretion of root exudates in response to Fe-deficiency

Under limiting Fe conditions, it is presumed that secretion of iron-binding root exudates may be essential for the development of Fe efficiency in plant species (Rodríguez-Celma et al., 2013). The roots of some Strategy I species secrete organic acids, riboflavin, flavins, flavonoids and phenolic compounds in response to iron deficiency, (Rodríguez-Celma et al., 2013; Abadía et al., 2011; Donnini et al., 2011). Hindering the exudation of flavins in Fe-starved *Medicago truncatula* seedlings led to a remarkable decrease in leaf Fe concentration, a phenomenon implicating a role of flavins in the Fe acquisition mechanism (Rodríguez-Celma et al., 2013). Growth and other typical phenotypic characteristics of mutants defective in phenolic synthesis were slightly restored when mutant seedlings were grown on low Fe medium (Rodríguez-Celma et al., 2011, 2013). Phenolic content increases in response to Fe-deficiency stress conditions and various environmental factors (Rodríguez-Celma et al., 2011, 2013). Research on the secretion of phenolics by red clover roots presented proof of a role of Fe deficiency-induced production of phenolics in Fe acquisition (Jin et al., 2007). Phenolic compounds can act as antioxidants by chelating metal or scavenging ROS (Perron and

Brumaghim, 2009). Aside their chelating property, phenolics are considered to be involved in the reduction of Fe^{3+} (Rodríguez-Celma et al., 2011).

1.6.5 Oxidative stress responses to iron supply in Strategy I plants

Reactive oxygen species (ROS) are constantly generated under normal physiological conditions in living organisms as a consequence of aerobic metabolism and cell signalling mechanisms (Bhaduri and Fulekar, 2012; Rout et al., 2015). In plants, ROS are produced mainly in chloroplasts, mitochondria and peroxisomes (Bhaduri and Fulekar, 2012). Superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), lipid peroxyl ($LOO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($O_2^{\bullet}$) are some of the major ROS in living systems. Excess production and/or lack of regulation of ROS normally lead to oxidative stress which can have deleterious effects such as DNA and RNA damage, oxidation of proteins, membrane lipid peroxidation and may eventually result in cell death (Panda et al. 2012; Bhaduri and Fulekar, 2012; Perron and Brumaghim, 2009). Oxidative stress resulting from the formation of ROS causes release of iron from proteins. Superoxide and H_2O_2 produced within cells can generate reactive hydroxyl radicals through Fe^{2+} and Fe^{3+} catalysed reactions (Kim and Guerinot, 2007). H_2O_2 is reduced by Fe^{2+} resulting in the formation of $OH^{\bullet}$ through Fenton- type reactions (Perron and Brumaghim, 2009). Excess and/or non-protein bound Fe can lead to iron-mediated oxidative DNA damage by hydroxyl radical (Perron and Brumaghim, 2009). High Fe concentrations and ferritins have been found to be associated with ROS production and oxidative stress (Reyt et al. 2015; Ravet et al., 2009; Briat et al., 2010).

Antioxidant enzymes elevate their activities to prevent oxidative stress in plants through free radicals and peroxides scavenging (see Table 1). Iron is necessary for the function of many antioxidant enzymes because Fe is present in the heme group of these proteins and serves as a catalyst of electron transfer reactions (Tewari et al., 2013; Zamboni et al., 2012). Salama et al. (2009) suggested a reduced functional Fe in plants under iron deficiency and a decrease in the activities of iron-containing antioxidative enzymes such as APX, POD and CAT. Excess iron growth conditions resulted in elevated antioxidant activities (see Table 1) in Arabidopsis plants (Ravet et al., 2009) and *Withania somnifera L.* (Rout et al., 2015). Similarly, increases in antioxidant enzyme activities in response to Fe stress have been observed in, *Triticum aestivum* and *Bacopa monnieri.*

Table 1. Antioxidant enzymes activity and expression in plants iron stress conditions.

Plants	Fe stress	Antioxidant enzyme	Enzyme activity	Reference
Potato	Fe deficiency	CAT, POD	Decreased	Chatterjee et al. (2006)
Sunflower	Fe deficiency	APX , POD	Decreased	Ranieri et al. (2001)
		SOD	Increased	
Sugar beet	Fe deficiency	GR	Increased	Zaharieva and Abadía (2003)
		APX	Decreased	Zaharieva et al. (2004)
Arabidopsis	Fe deficiency	APX	Decreased	Ramirez et al. (2013)
		CAT	not modified	
Maize	Fe deficiency	APX, POD, CAT	Decreased	Sun et al. (2007)
		SOD, GR	Increased	
Tomato	Fe deficiency	CAT, SOD, POD	decreased	Zamboni et al. (2012)
Flax	Fe deficiency	APX, POD, CAT	Reduced	Salama et al. (2009)
		SOD	Increased	
Mulberry, maize and cauliflower	Fe deficiency	CAT, POD, APX	Decreased	Tewari et al. (2005)
		SOD	increased	
Citrus, pea, tobacco	Fe deficiency	Fe-SOD	decrease	Sevilla et al. (1984); Iturbe-Ormaetxe et al. (1995); Kurepa et al. (1997)
Pea	Fe deficiency	CAT, POD, APX	decreased	Iturbe-Ormaetxe et al. (1995)
		Cu/Zn-SOD	increased	
Winter Cherry	Excess Fe	CAT, SOD, GPX	increased	Rout et al. (2015)
Water hyssop	Excess Fe	SOD	increased	Sinha and Saxena (2006)
Sweet potato	Excess Fe	SOD , APX	increased	Adamski et al. (2012)
Arabidopsis	Excess Fe	APX, SOD,CAT	increased	Ravet et al. (2009)
		CAT	increased	Reyt et al. (2015)
Wheat	Excess Fe	CAT, SOD,POD	increased	Li et al. (2012)

CAT: catalase; POD: peroxidase; APX: ascorbate peroxidase; SOD: superoxide dismutase; GR: Glutathione reductase

1.7 Improvement of iron uptake and nutrition in crops

1.7.1 Fertiliser and cultural management practices

A number of approaches have been employed to deal with the problem of Fe chlorosis. Iron fertilisers only provide a short-term increase in Fe concentration. Inorganic Fe fertiliser ($FeSO_4$) application on Fe-deficient soils has been found to be mostly ineffective since the applied Fe is converted into unavailable Fe (III) forms (Hansen et al., 2006; Zuo and Zhang, 2011). When $FeSO_4$ is incorporated into calcareous soils, it reacts with $CaCO_3$ to form Fe oxides. These oxides are highly insoluble and are less available for plant uptake. Though foliar application of Fe can make Fe readily available to plants, this technique can hinder the plant's natural ability to overcome Fe deficiency (Römheld and Marschner, 1986). To have a marked impact on crop yields foliar Fe sprays need to be applied repeatedly making this management approach expensive and impractical especially for low value crops (Hansen et al., 2006).

The application of synthetic Fe chelates (Fe-EDTA or Fe-EDDHA) have shown promising results compared to inorganic Fe salts ($FeSO_4$) but they are costly and success is limited (Zuo and Zhang, 2011). EDDHA (ethylenediamine-N, N'-bis (2-hydroxyphenylacetic acid) is the main chelator shown to be effective in Fe-deficient soils (Abadía et al., 2004; Hansen et al., 2006). Fe-EDDHA, however, is costly and an alternative only for high value crops (Hansen et al., 2006). Since synthetic and negatively charged chelates can be repelled by a negatively charged soil, the usefulness of an Fe chelate in enhancing soil Fe availability is limited (Abadía et al., 2004; Hansen et al., 2006). Sometimes, excessive irrigation and rainfall may cause the chelate to leach from the root zone (Hansen et al., 2006). The amount and type of clay and salt in the soil affects the effectiveness of synthetic chelates (Barton and Abadía, 2006; Hansen et al., 2006).

Increasing drainage in poorly drained soils is effective in improving compaction by reducing oxygen/moisture ratio and root growth which subsequently improve Fe availability (Hansen et al., 2006; Barton and Abadía, 2006). Tillage of compacted soils has also been found to be valuable in temporarily enhancing Fe availability (Barton and Abadía, 2006). Organic matter is useful in preventing Fe deficiency because it serves as a good source of Fe but when applied in large quantities can cause "loosening" of the soil (Hansen et al., 2006).

Improving Fe uptake in plants by lowering soil pH to increase the solubility of soil Fe requires an enormous amount of acidifying substance making this option cost ineffective except for high value crops where cost is not an issue. The application of acidified irrigation

water decreases the bicarbonate concentration and gradually dissolves soil carbonates, conditions which result in improved Fe nutrition (Hansen et al., 2006).

Virtually all of the fertiliser and cultural management practices have been shown to correct Fe deficiencies to some extent but many are unpredictable, impractical and/or too expensive (Hansen et al., 2006). Additionally, Fe limitation cannot simply be overcome using iron-containing fertilisers since low iron bioavailability is not a problem of abundance but rather of solubility (Hell and Stephan, 2003).

1.7.2 Enhancement of iron bioavailability and content

Iron nutrition in crops can be improved by enhancing Fe acquisition by the plant or by modifying Fe-storage proteins in seeds to increase bioavailable Fe (Cianzio et al., 2006). Ways by which iron can be made readily available include:

1) Lowering or removal of specific anti-nutritional factors/iron inhibitors (e.g. phytate, tannic acid). Tannic acid has a galloyl content which forms a complex with iron and as a result, inhibits its absorption from food (Vasconcelos and Grusak, 2006). Tannins are found in the buds, leaves, roots and seeds of angiosperm and gymnosperms species. Phytate is an anti-nutritional compound in many cereal crops. It can bind to iron and render it unavailable to plants (Vasconcelos and Grusak, 2006; Hell and Stephan, 2003). Incorporation of phytases into plants breaks down phytate to release the bound iron and thereby increase iron bioavailability. This technique has been used by Lucca et al. (2002): they introduced a phytase from *Aspergillus fumigatus* into rice endosperm to enhance iron bioavailability. Low seed-phytate mutants of some crop species were identified to have increased zinc bioavailability (Vasconcelos and Grusak, 2006).

2) Increasing or incorporating an iron absorption promotive compound, ascorbic acid enhances iron bioavailability through its ability to reduce Fe^{3+} (Vasconcelos and Grusak, 2006). The amino acid, cysteine, can act as an enhancer of iron absorption: over-expression of an endogenous cysteine-rich metallothionein-like protein in transgenic rice carrying the ferritin gene led to a sevenfold increase in the content of cysteine residues (Lucca et al., 2002).

1.7.3 Cultivar selection of Fe-efficient plants

The identification and isolation of IDC-tolerant species can lead to the enhancement of crop productivity in calcareous soils. Development of IDC-tolerant cultivars presents the most

important method of controlling Fe chlorosis development in field crops (Zhu et al., 2015; Cianzio et al., 2006; Helms et al., 2010; Vasconcelos and Grusak, 2014). Classical breeding approaches were employed to breed for plants adapted to high lime soils by identification of genotypes resistant to IDC (Cianzio et al., 2006). After crossing resistant genotypes with susceptible ones, IDC-resistant genotypes possessing agronomically-important traits were selected. However, this technique takes years of screening under controlled and field conditions.

In vitro identification and selection of plants tolerant to chlorosis requires a considerably shorter time compared to selection using *in situ* breeding programs (Mahdavikia and Mahna, 2012). Using *in vitro* culture, somaclonal variation enables the production of novel clones with desirable agronomic characters and serves as a significant adjunct for plant improvement (Thieme and Griess, 2005; Larkin and Scowcroft, 1981**;** Karp, 1991). The selection of Fe-efficient (chlorosis resistant) cell lines through *in vitro* culture technique was suggested by Ross (1986). The selection and use of Fe-efficient plants is generally the best management approach for alleviating IDC and for improving crop production (Zhu et al., 2015; Hansen et al., 2006; García-Mina et al., 2013; Vasconcelos and Grusak, 2014). Sain and Johnson (1984) reported on soybean (*Glycine max*) genotypes differing in resistance to iron deficiency. Naik et al. (1990) generated Fe-efficient sugarcane lines while Bacaicoa and García-Mina (2009) selected Fe-efficient cucumber plants following *in vitro* exposure of their cell cultures to Fe deficiency conditions. *In vitro* techniques have been successfully employed in the selection and characterisation of iron efficiency in pear (Dolcet-Sanjuan et al., 1992) and selection of Fe-deficiency resistant quince (Marino et al., 2000; Muleo et al., 1995). Bavaresco et al. (1993) screened four grapevine genotypes with different tolerance to lime-induced chlorosis and identified genotypes with a higher tolerance to chlorosis. Tangolar et al. (2008) screened for iron-deficiency tolerant grapevine. Serpil et al. (2008) used different concentrations of FeNaEDTA to screen and assess grape cultivars tolerant and susceptible to sodium bicarbonate-induced chlorosis for planting on alkaline soils. Vasconcelos and Grusak (2014) identified novel sets of Fe-efficient cell lines in soybean and confirmed their tolerance to IDC under laboratory conditions.

1.7.4 Iron biofortification strategies

Biofortification capitalises on food staples that predominate in the diets of people worldwide to develop micronutrient-dense germplasm that can be distributed internationally (Nestel et

al., 2006; Bouis, 2003, 2000). Biofortified crops can complement existing approaches by playing a role as an integral part of food system interventions to sustainably provide micronutrients to people worldwide. It is a more sustainable solution than supplementation or fortification (Haynes et al., 2012**;** Poletti and Sautter, 2005; Bouis, 2003, 2000) and economically practicable (Bouis, 2002; Haynes et al., 2012). Conventional plant breeding approaches to biofortification have been used in various plants and have wide public acceptance (Saltzman et al. 2013). Recurrent costs are low and represent a small portion of a public health intervention through supplementation (Bouis, 2003; Poletti and Sautter, 2005; Carvalho and Vasconcelos, 2013).

Biofortification has been employed to obtain iron-enriched crops that can the boost the nutritional status of people. Traditionally bred iron biofortified products currently released and found to be effective in improving micronutrient levels in people include: iron-rich (ICTP 8203-Fe) pearl millet in 2012, beans with higher iron levels in Rwanda in 2012, high-iron and zinc cowpea varieties (Pant Lobia-1 and Pant Lobia-2) released by the Uttarakhand Government in 2008 and 2010 respectively and iron-rich rice released in China in 2011 (Saltzman et al., 2013). Potato has been given little attention in iron biofortification projects despite its good nutritional qualities, low iron content and increased global consumption; efforts to produce micronutrient-dense potatoes are still in their very early stages.

Genetic engineering strategies are employed especially where there is insufficient genetic variation to reach target nutrient levels through traditional breeding (Mayer et al. 2008; Ihemere et al., 2012). Iron-rich transgenic rice (Goto et al., 1999; Lucca et al., 2001, 2002; Wirth et al., 2009) and cassava (Ihemere et al., 2012) have been successfully produced. The transgenic rice lines obtained through the incorporation of a ferritin gene from common bean into rice had double the amount of iron in seeds compared to controls (Lucca et al., 2001, 2002). Khokan et al. (2009) developed a protocol for transgenic potato with ferritin gene and suggested that potato with enhanced iron content may be developed by genetic transformation. Biofortification through genetic engineering and nanotechnology is expensive, and the resulting products require long and extensive biosafety regulatory approval, take several years to be commercialised and most possibly face challenges of low consumer acceptance.

Table 2. Interventions and management strategies for amelioration of Fe deficiency in plants.

Strategies	Effectiveness and/ or Benefits	Limitations	Reference
Inorganic fertilizers ($FeSO$, $[Fe_2(SO_4)_3$)	very limited effect , short-term increase in Fe concentration	frequent application, high dosage requirements, Fe^{2+} is converted into unavailable Fe^{3+} adverse environmental impacts	Abadia et al. (2011); Hansen et al. (2006); Shenker and Chen (2005); Zuo and Zhang 2011).
Synthetic Fe-chelates (e.g FeEDTA, FeEDDHA, Fe-DTPA)	positive agronomic outcomes, highly stable and soluble, enhances soil Fe availability effectiveness depends on soil pH	require repeated application, expensive economically impractical, limited success, easily leached	Covarrubias et al. (2014); Zuo and Zhang (2011); Shenker and Chen (2005)
Natural Fe-complexes fertilizers (e.g. humates, lignosulfonates, amino acids, gluconate, citrate)	relatively ineffective, enhances growth response of plants	reduced Fe availability due to metal- and ligand-exchange reactions unpredictable	Shenker and Chen (2005) ; Abadia et al. (2011)
Slow-release Fe fertilizers: Fe-phosphate vivianite ([Fe3(PO4)2.8H2O]) and Fe(III) phosphates	effective in preventing chlorosis , long-term, improved fertilizer use efficiency, low dosage requirements, negligible environmental pollution	high cost, low solubility, fast precipitation, disparity in nutrient release and plant uptake rates	Eynard et al. (1992); Chandra et al. (2009); Abadia et al. (2011)
Foliar iron sprays and tree injections	beneficial effects on small scale	can hinder plant's natural ability to overcome Fe deficiency; expensive, repeated use, short-term, impractical for large scale use or low value crops	Hansen et al. (2006); Zuo and Zhang (2011)

Table 2 continued.

Strategies	Effectiveness and/ or Benefits	Limitations	Reference
Antagonistic nutrient management	improves Fe availability	difficulty in regulating soil nutrients, dependent on plant species and soil conditions, unpredictable, impractical	Shenker and Chen (2005)
Intercropping with graminaceous species	enhance leaf chlorophyll index, prevent Fe chlorosis, increase Fe content of plants	long term, varies among plant species, unpredictable	Zuo and Zhang (2011); Covarrubias et al. (2014)
Acidified or regulated irrigation	easy-to-apply, decreases soil pH, increases Fe availability	cost ineffective, long-lasting, soil acidification is practically impossible on large scale	Shenker and Chen (2005) Hansen et al. (2006); Zuo and Zhang (2011)
Tillage of compacted soils	temporarily enhances Fe availability	laborous, time consuming, temporal	Barton and Abadía (2006)
Field selection of Fe-defi ciency chlorosis tolerant cultivars	high tolerance to iron-deficiency conditions	laborous, time consuming	Helms et al. (2010); Shenker and Chen (2005); Goos and Johnson (2000)
transgenic rice with higher amounts of phytosiderophores	enhanced tolerance of plant to low iron, greater grain yields	long-term process, costly, high expertise required	Takahashi et al. (2001)

Table 2 continued.

Strategies	Effectiveness and/ or Benefits	Limitations	Reference
Biofertilization with microbial siderophores	supplies Fe to plants, efficient Fe mediators for plant roots in soil.	low levels of siderophores in natural habitats, short half-life, limited detectability of siderophores	Shenker and Chen (2005)
Transgenic rice and soybean expressing *FRO*	reduced chlorosis, higher Fe-uptake rate, enhanced FRO activity, increased chlorophyll content	long-term process, expensive, requires high expertise	Ishimaru et al. (2007); Vasconcelos et al. (2006)
Fe biofortified transgenic rice cassava and rice	tolerance to Fe-deficiency, efficient uptake of iron, increase in Fe levels,	long-term process, costly, requires high expertise	Lucca et al.(2002); Masuda et al. (2013); Ihemere et al. (2012)
Breeding and selection of Fe-efficient and chlorosis resistant plants	alleviates chlorosis, increase yields, allows crop production on marginal lands, reduces need for Fe-fertilization, long lasting, resistance can be hereditary and transmitted to the progeny	long-term process	Naik et al. (1990); Marino et al. (2000); Shenker and Chen (2005); Tangolar et al. (2008); Palombi et al.(2007)

1.8 Plant tissue culture-based *in vitro* selection

Plant tissue culture (PTC) technology plays an important role in the advancement of plant science research, food production and synthesis of secondary products. Potato improvement by means of traditional breeding and field selection programmes for identifying superior genotypes is a lengthy process that is labour intensive, unpredictable and burdensome (Rai et al., 2011). PTC has become known as a cost-effective and practical means of developing stress-tolerant plants. Disease-free plants can be propagated through *in vitro* plant cell culture and multiplied rapidly as ‘‘cleaned stock’’ for healthy seed tubers production at a low cost (Lutaladio and Castaldi, 2009). *In vitro* propagation and microtuberisation of potato makes it possible for large amounts of materials to be assessed regardless of the crop season because, complex organ-organ and plant-environment interaction as well as the level of stress can be accurately and conveniently controlled. It does not require extensive expertise and/or prior knowledge of the genetic make-up or sequence of a gene associated with the desired trait. *In vitro* selection is an alternative tool to complement conventional field selection methods of acquiring valuable agronomic traits (Rai et al., 2011).

1.8.1 Somaclonal variation (SV)

Somaclonal variation constitutes the genetic or epigenetic modifications that occur in cells or plants propagated from a common donor clone as a result of *in vitro* tissue culture cycle (Lestari, 2006; Larkin and Scowcroft, 1981). Somaclonal variation can occur spontaneously in plant tissue or cell cultures, may be induced or increased by means of mutagen treatments and can be introduced during cell fusion or transformation (Larkin and Scowcroft, 1981; Widholm, 1989). With SV, changes in agronomically desirable traits can occur at a high rate and certain novel variants that may not be attained by conventional breeding can be obtained (Phillips et al., 1994; Widholm, 1989). However, the genetic stability of the variation in nature may be unpredictable and variation may not occur for complex agronomic traits (Jain, 2001).

1.8.2 Sources of somaclonal variation

Rate or extent of somaclonal variation is strongly influenced by genome composition (Sahijram et al., 2003) and the tissue culture environment (Martin et al., 2006). The possible sources of somaclonal variation include plant genotype, polyploidy level, explant type, culture medium, growth regulators, age of the donor plants, *in vitro* culture period and extent

of deviation from organised growth (Bairu et al., 2011; Bordallo et al., 2004; Jain, 2001; Karp, 1995; Jain et al., 1998). The type and frequency of SV is highly controlled by plant genotype since some plant species are reported to produce a wide range of somaclonal variation (Bairu et al., 2011; Jain, 2001). The source of the tissue used in the *in vitro* culture can influence the nature and occurrence of somaclonal variation. Leaves, roots and stems generate more variants than explants from axillary buds and shoot tips because they (buds and shoot tips) are well differentiated (Sharma et al., 2007; Karp et al., 1989).

1.8.3 *In vitro* culture period and somaclonal variation

Over the years there has been conflicting reports on the rate of somaclonal variation in relation to length of the culture period. Although it was originally put forward that increase in culture period or age was directly proportional to the extent of somaclonal variation generated, some studies detected that older cultures produced regenerants with less somaclonal variation or that plants could not be regenerated from such cultures (Wang and Wang, 2012). In many cell suspension and callus cultures, SV was suggested to occur and /or accumulate with culture age (Rodrigues et al., 1998; Petolino et al., 2003).

An equation, $\%V=[1-(1-p)^n] \times 100$, for computing somaclonal variation percentage was proposed by Coˆte et al. (2001) to describe the connection between SV and culture period. In this statistical model, n denotes the number of multiplication cycles and %V, the percentage of variant. According to this model, variation increases as the culture ages as was indicated in some reports (Phillips et al., 1994; Bouman and DeKlerk, 2001; Peredo et al., 2006). However, contrasting findings were reported in other studies (Lo´pez et al. 2010; Hao and Deng, 2002) where SV was observed to gradually increase to a peak and thereafter, decrease in cells and plants cultured for extended periods. The model has limited application owing to the fact that the complexity of biological systems was not factored in.

Wang and Wang, (2012) described the relationship connecting the culture period and SV frequencies in calli or regenerants from an evolutionary perspective. They proposed three *in vitro* selection models (V-win, T-win and draw) with three major classifications of calli (V-callus, T-callus and D-callus) and hypothesised an *in vitro* callus selection based on natural selection. True-to-type and 'wild-type' cells often divide and grow more rapidly than the deleterious cells (Wang and Wang, 2012). It was suggested that differentiation bottleneck (D-bottleneck) can be used to deduce or explain the loss of the regenerability in cultures kept over extended period of time. Older callus did not reveal a corresponding increase in the

frequency of SV due to D-bottleneck (Wang and Wang, 2012). The authors proposed a SV 'threshold' based on the premise that plant SV is finite and speculated that when SV is above the threshold no regenerants would be acquired. Calli with intense SV can lose vigour and regenerability over a certain threshold (Kaeppler et al., 2000; Wang et al., 2011).

1.8.4 Somaclonal variation based on epigenetics

Epigenetic traits are linked to modifications in regulation of gene expression or biochemical events which are generally neither expressed in regenerated plants nor heritable through meiosis (Bairu et al., 2011; Wang and Wang, 2012). Reprogramming of plant regenerants upon exposure to traumatic changes in a tissue culture environment, produce epigenetic variations (Bairu et al., 2011; Jain, 2000, 2001; Meredith, 1984). Epigenetic changes are usually not transmitted by sexual reproduction; they are reversible, not meiotically heritable and termed as 'variation' (Bairu et al., 2011; Wang and Wang, 2012; Coˆte et al., 2001). However, epigenetic responses can persist throughout mitoses, are stable and independent of the event that caused the change (Berlin and Sasse, 1985). Mitotically stable epigenetic alterations to selective agents can obscure the selection of mutants that can be meiotically inherited (Tal, 1994). Similar to genetic SV, epigenetic SV in calli can be transmitted to regenerated plants (Fojtova et al., 2003; Kaeppler et al., 2000).

The silencing of genes, alterations in DNA methylation, activation of transposable elements and retro transposons lend credence to a role of epigenetic mechanisms in somaclonal variation (Bairu et al., 2011). DNA methylation causes instability in somaclones (Jain et al., 1998; Bairu et al., 2011). Plant tissue culture-derived alteration in DNA methylations account for a high percentage of epigenetic variations in a number of plant species (Guo et al., 2007; Gao et al., 2010; Lo´pez et al., 2010; Wang et al., 2012). Vázquez, (2001) reported that methylation in cells persisted through meiosis, can be passed on to offspring and sometimes stable through generations. Kaeppler et al. (1998) argued that transposable elements could be responsible for a small fraction of tissue culture-induced variation. Transposon activity is associated with changes in DNA methylation in plants (Peschke et al., 1991; Ngezahayo et al., 2009). Activation of transposable elements can result in insertion of a transposon into a nearby gene to cause phenotypic alterations (Yu et al., 2007) detectable in regenerants and regenerate-derived progeny (Ngezahayo et al., 2009; Peschke et al., 1991). Induction of transposable elements can be responsible for modification of gene regulation and point mutations (Vázquez, 2001).

1.8.5 Somaclonal variation based on genetics

Genetic alterations are permanent, heritable, can be sexually transmitted and persist in the progeny of regenerated plants (Bairu et al., 2011; Roy and Mandal, 2005; Kaeppler et al., 2000; Rietveld et al., 1993). A variation that is meiotically stable is referred to as a 'mutation'. Such 'mutations' are usually heritable and the altered phenotypes can be passed on to progeny. Genetic variations take place in calli, undifferentiated cells, protoplasts and regenerated plants (Pérez-Clemente and Gómez-Cadenas, 2012**)**. Somaclonal variations that remain stable are often either due to the expression of an existing variation in the source plant or to mechanisms that result in genetic variation (Larkin and Scowcroft, 1981). Genetically stable and heritable somaclonal variants can be used to investigate mechanisms of genetic change and are useful in plant breeding programs.

Changes in the chromosome number and structure, gene activation or inactivation point mutations and cytogenetic abnormalities constitute the major causes of genetic modifications and are regularly found in regenerated plants and their progeny (Pérez-Clemente and Gómez-Cadenas, 2012; Wang and Wang, 2012; Bairu et al., 2011). Phenotypic changes in regenerated plants and their progeny are mainly due to point mutations and are recessive (Phillips et al. 1994). Single gene mutants such as dwarfs, seed traits, chlorophyll deficiency and necrotic leaves were discovered in maize (Phillips et al., 1994). Cytogenetic aberrations include chromosome rearrangements (deletions, insertions, inversions, duplications and translocations) and ploidy changes. In tissue culture, DNA methylation or the regulation of cell cycle can be altered and result in chromosome breakage and its consequences (Phillips et al., 1994; Jain, 2001). The extent and frequency of chromosomal instability is influenced by the plant species and age of callus. Increase in callus age is proportional to increase in chromosomal aberrations but in maize callus, age had no influence on chromosomal changes (Jain, 2001).

In some instances, even though the selected desirable trait is a result of a stable genetic change, the trait is not expressed in regenerated plants because a gene that is developmentally regulated may be silent in the appropriate cells in the plant (Meredith, 1984; Berlin and Sasse, 1985). This implies that rare mutants may not always be expressed in plants owing to the developmental complexity of higher plants (Meredith, 1984; Berlin and Sasse, 1985).

1.8.6 Identification and characterisation of somaclonal variants

Dissimilarity in leaf morphology, plant stature and pigmentation abnormality are used to characterise variants. Dwarf off-types in banana were identified by visually examining the stature and leaf index (Rodrigues et al., 1998). Zaid and Al Kaabi (2003) reported morphological traits such as leaf whitening and variegation, excessive vegetative growth and bastard offshoots in date palm somaclonal variants. Many stable potato clones with major diversity in growth pattern, yield and vigour, maturation date, tuber size, number, skin colour and shape and disease response have been identified (Shepard et al., 1980; Karp et al., 1989). Slow growing dwarf dihaploid potato mutants with characteristics of short internodes, dark green leaves, long-day adaptation and compact and ball-shaped appearance in tetraploid were discovered by Valkonen et al. (1999). They determined dwarfism by a recessive Pito gene. Morphological characterisation however, may not be an exact mirror of genetic change because plant morphology is usually influenced by environmental factors (Bairu et al., 2011).

Various biochemical tests have been used to make a distinction between somaclones. Cell lines with high yields of secondary products such as steroids, alkaloids, flavourings and terpenes have been selected (Collin and Dix, 1990). Such variants are valuable in the enhancement of the study of biochemical and developmental pathways. Pineapple variants were detected to have a considerable decrease in chlorophyll content compared to normal regenerants (Mujib, 2005). Photosynthesis rates were found to be lower in plants exposed to salinity stress (Rai et al., 2011; Parida and Das, 2005). Wang et al. (2007) observed significant variation in total carotenoid levels in sweet potato amongst somaclonal variants and normal cultures. Peroxidation of membrane lipids measured as the level of malondialdehyde produced is used as an indicator of oxidative damage in plants under salinity stress (Demiral and Turkan, 2005). Selected tolerant callus lines of *Solanum tuberosum* subjected to NaCl showed an increase in lipid peroxidation (Queiros et al., 2007). Enzyme analyses of superoxide dismutase, peroxidase and malate dehydrogenase were carried out to determine variation in beans (Gonza´lez et al., 2010), sugarcane (Srivastava et al., 2005). Mechanisms of tolerant plants include ion homeostasis, antioxidant enzyme defence and osmotic adaptation (Rai et al., 2011; Kumar et al., 2010). High concentrations of iron in seeds were recorded in plants with increased resistance to Fe deficiency chlorosis (Vasconcelos and Grusak 2014).

Transcriptome studies on modifications and quantification of gene expression (by RT-qPCR) can be beneficial for identifying plant variants or mutants. RT-qPCR is a three-step technique for the quantification of mRNA: (i) conversion of RNA into cDNA, (ii) the

amplification of cDNA and (iii) the measurement of amplification products in real time (Nolan et al., 2006). The frequently used normalisation strategy involves standardisation to a constitutively expressed reference gene (e.g. 18S and 28S rRNA, or β-actin) as the internal control (Andersen et al., 2004; Song et al., 2012). Expression profiles and data on iron uptake genes from different species analysed by quantitative RT–PCR and microarray, have been discussed in section 1.6.1. Using these molecular techniques, genes that are differentially regulated by iron irrespective of growth conditions, or the age of plants have been identified (Ivanov et al., 2012). The authors reported on the genetic control of iron acquisition in Strategy I plants revealing novel potential gene interactions and reliable iron-deficiency marker genes.

At the molecular level, specific sequence changes can be examined by random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism, (AFLP), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) methods (Gao et al., 2010; Wang et al., 2011). Restriction enzyme analysis based on the cutting of specific DNA sequence motifs has been employed to study molecular variation relating to DNA and protein level in tissue cultured plants. Modifications in restriction pattern emerged as altered fragment size (Kaeppler et al., 1998). RAPD technique can detect polymorphisms among the amplification products displayed as visible bands on ethidium bromide-stained agarose gel. With RAPD analysis, single short primers (genetic markers) of arbitrary nucleotide sequence are used to amplify segments of target genomic DNA (Bairu et al., 2011). Palombi et al. (2007) detected polymorphisms in chlorosis tolerant pear clones relative to the mother clone using RAPD. Their studies revealed genetic variability among the 11 regenerated clones and between them and the donor clone. Microsatellite marker techniques are sequence specific, require less amount of DNA and have been used to assess genetic stability of *in vitro* cultured plants as in rice (Gao et al., 2009), grapevine (Welter et al., 2007), sorghum (Zhang et al., 2010), and sugarcane (Singh et al., 2008).

1.9 *In vitro* selection of desirable plant traits

Selection *in vitro* is a powerful tool since a large population of cells can be utilised and screened within a short duration in a relatively little space. The effectiveness of *in vitro* selection is due to its potential to generate a plant with the desired character of interest (Lestari, 2006; Rai et al., 2011). The best strategy towards this is to manipulate somaclones

with an appropriate selection pressure (Rai et al., 2011; Pérez-Clemente and Gómez-Cadenas, 2012; Lestari, 2006). A suitable selection agent enables the preferential survival and/or growth of variant cells and plants with desirable phenotypes (Pérez-Clemente and Gómez-Cadenas, 2012**;** Rai et al., 2011). Desirable and genetically stable traits obtained under selection pressure with negligible environmental interaction, can complement field selection (Jain, 2001; Rai et al., 2011). The success of *in vitro* selection technique in acquiring tolerant plants depends on the desired character to be inherited, the convenient utilisation of *in vitro* selection system, high cell variation and the mode of regeneration of tolerant cell lines (Lestari, 2006).

1.9.1 *In vitro* selection in potato

Phenotypic changes arising from physiological, epigenetic or genetic alterations during the cell culture and regeneration of potato from leaf protoplasts have led to the selection and recovery of a number of novel phenotypes (Shepard et al., 1980; Karp, 1991). With regards to improvement in potato production, cell selection has mainly been applied in the selection of disease resistant potato plants by exposing cultured cells to filtrates of pathogens or pathotoxins. Wilson et al. (2009, 2010) identified and selected common scab disease resistant potato (cv. Iwa) clones through somatic cell selection. Hiltunen et al. (2006) observed a reduced occurrence of scab lesions on tubers of cultured potato seedlings that developed increased resistance to thaxtomin A (phytotoxin from *Streptomyces* species). Tubers susceptible to common scab disease caused by *Streptomyces scabies* develop deep-pitted surface lesions which result in loss of quality, yield and economic returns. More research on the selection of potato cell lines resistant to thaxtomin A has been carried out and plants with enhanced resistance to common scab recovered (Tegg et al., 2013; Wilson et al., 2009, 2010). Disease resistance in the selected rare clones was stable and maintained in field trials (Tegg et al., 2013; Wilson et al., 2010). Potato plants with improved tolerance to salt were identified using somatic cell selection (Queiros et al., 2007).

1.9.2 Callus culture system for *in vitro* selection

Selection for variant cells of interest can be done with callus, suspension cultures, plated cells, somatic embryos and protoplasts. The objective of the selection programme usually dictates which culture system is most appropriate to use. Each culture system has its merits and demerits which can be exploited to provide a positive outcome in the design of selection

and screening schemes. A breakdown in the organisational structure of a plant *in vitro* which results in somaclonal variation (Bairu et al., 2011) is dependent on the culture system used. Careful optimisation of a chosen culture system is valuable for the recovery of variant cells.

Plants develop callus, a mass of disorganised cells, in response to various biotic and abiotic stimuli. Callus formation is as a result of dramatic changes in the metabolism of the cells leading to the breakdown of intercellular, physical and chemical communication (Aitchison et al., 1978; Lindsey and Jones, 1992). Callus cultures are suggested to induce unorganised growth and to produce instability in plant tissue at a considerably high rate (Bairu et al., 2011; Vázquez, 2001). A high degree of cellular disorganisation in a tissue culture system leads to an increased probability of obtaining mutants (Arau´jo et al., 2001; Cooper et al., 2006). Callus can be induced *in vitro* by the culture of plant explants (e.g. leaf, stem or root) in a suitable nutrient medium containing plant growth regulators.

Callus culture systems are the most convenient for use in screening and selection programmes and have higher mutation rates (Vázquez, 2001). Callus cells are in close contact with each other thus, it is highly likely to recover a mixed population of resistant and sensitive cells. The close connection between callus cells allow cross-feeding and promote escape from the selection (Meredith, 1984; Berlin and Sasse, 1985). Thus with callus selection, variant cells expressing a trait of interest can easily go undetected if they are within the vicinity of non-growing cells (Meredith, 1984**)**. However, repeated culture of callus cells on inhibitory concentrations of the selective compound or condition can eliminate the sensitive cells (Berlin and Sasse, 1985). Friable callus cells can often be finely dispersed on selective agar medium and this reduces the problem of escapes (Berlin and Sasse, 1985).

1.9.3 Visual selection in callus cultures

Callus can be assessed visually for differences in pigmentation by the naked eye, with an ultraviolet-lamp for fluorescent spots or for alterations in growth characteristics (Duncan and Widholm, 1990). Pigment variants can be simply identified by visually scanning cultured cells. Altered pigmented areas of callus are easy to detect growing callus regions with intense colour provides a means for the selection of variant lines high in pigmentation (Berlin and Sasse, 1985). High anthocyanin-containing sweet potato (Nozue et al., 1987) and *Bubleurum falcatum* (Hiraoka et al., 1986) callus cultures were identified by visual selection. Accumulation of anthocyanin (Chaudhary and Mukhopadhyay, 2012; Ananga et al., 2013) and loss of chlorophyll pigmentation (Svab and Maliga, 1986) were observed and visually

selected. Visual screening of callus for cells with altered characteristics can be inefficient and has a limited scope (Meredith, 1984; Berlin and Sasse, 1985). A reason for this is the probable recovery of a mixed population of resistant and sensitive cells due to the uneven exposure of cells of a callus to the selective agent in a culture medium (Berlin and Sasse, 1985; Meredith, 1984).

1.9.4 Resistance selection in callus cultures

Resistance selection can be used in conditions where the required phenotype has a selective advantage over wild type cells. Resistant cells capable of growth in the presence of the inhibitor can be identified and isolated. Resistance selection using callus cultures involves the excision of small pieces of growing callus (selected cells) from surrounding dead cells. The selection procedure can be improved by subculturing minute pieces of callus in order to reduce the risk of formation of chimaeras (Collin and Dix, 1990). Callus pieces of 20 mg were used in the selection of salt tolerance in *Brassica napus* (Chandler and Thorpe, 1987) and 100 mg *Beta vulgaris* (Pua and Thorpe, 1986), respectively. About 10-20mg callus pieces were used in the selection of 5-methyl tryptophan resistant rice (Wakasa and Widholm, 1987).

Callus cultures are commonly used for resistance selection for a number of traits (see Table 3). NaCl-resistant tobacco (Nabors et al,.1980), sugarcane (Naik et al., 1990) and citrus (Ben-Hayyim and Kochba, 1982) plants have been successfully developed from callus-derived resistant lines. Ochatt et al. (1999) obtained salt-tolerant potato plants from stable salt-tolerant cell-lines. The selection strategies to isolate variants resistant to mineral stresses involve subjecting cells to a culture medium containing the mineral deficiency or toxicity of interest. Novel crop genotypes with resistance to mineral stresses can be produced by *in vitro* selection (see Table 3). Ni-tolerant lines were detected in callus cultures of *Setaria italica L.* (Rout et al., 1998). Lines resistant to Fe-deficiency have been determines in various plants as discussed earlier in this chapter (see section 1.8.3).

The stability of a selected trait in callus cultures can be maintained for a prolonged period on the selective medium (see Table 3). Bennetzen and Adams (1984) obtained cadmium-resistant *Lycopersicon* species that were stable over 30 cultures. Callus cultures of sweet potato selected for high anthocyanin levels were discovered to be stable after 35 subcultures and also in the absence of the selection pressure (Nozue et al., 1987). Salt tolerance in *Beta vulgaris* (Pua and Thorpe, 1986) and *Citrus aurantinum* (Ben-Hayyim et al.,

1985) was found to be sustained after nine months and one year respectively in culture under selective pressure.

1.9.5 *In vitro* selection schemes

Selection schemes can be designed to successfully recover plant genotypes resistant to various biotic and abiotic stresses. *In vitro* tissue culture- based selection schemes have been classified into two major categories: a selection pressure can be applied gradually in a step-wise fashion or suddenly as a shock treatment (Table 3). The choice of the most suitable selection approach depends on the selection objective of the research and the particular plant species under study (Table 3).

1.9.6 Direct selection

Direct selection involves the sudden exposure of cells to conditions that enable the survival of only a proportion of the population that can tolerate and are adapted to such conditions (e.gs. see Table 3). *In vitro* selection by sudden treatment was employed for isolation of resistance to herbicides (Jordan and McHughen, 1987) and salinity (Queirós et al., 2007; Chandler and Thorpe, 1987). Direct selection technique has also been used in the selection for metal tolerance: *in vitro* selection of copper-tolerant plants (Rout and Sahoo, 2007), zinc- and manganese-tolerant callus lines (Rout et al., 1999), chromium- and nickel-tolerant cell lines (Samantaray et al. 2001) and selection for iron efficiency (Dolcet-Sanjua et al., 1992; Naik et al., 1990). Rout et al. (1998) generated a Ni-tolerant cell line by the introduction of callus to media with high amounts of nickel (Ni). Some research findings suggest that short-term or one-step selection strategies can be used to prevent the development of epigenetically adapted cells (Rai et al., 2011; McHugen and Swartz, 1984; Chandler and Vasil, 1984; Tal, 1994). The use of direct selection in the isolation of salt-tolerant cell lines was deemed better compared to the gradual method of selection since direct selection conditions are similar to field conditions (Mc-Hughen and Swartz, 1984; Sabbah and Tal, 1990).

1.9.7 Gradual Selection

Multi-step (gradual) selection procedure involves transfer of cells to a culture medium of subsequently higher selective pressure or concentration (e.gs. see Table 3). Callus or cells are successively sub-cultured on media with varying (mostly increasing) concentrations of the selective compound or condition. Abdi et al. (2011) developed NaCl-tolerant lines from

selected callus line of *Tanacetum cinerariaefolium* through gradual increase in NaCl concentration. Increasing NaCl levels from low to high was found to be efficient in the isolation of NaCl-tolerant callus line. Koch et al. (2012) identified three imazapyr-tolerant genotypes through the stepwise increase in the imazapyr concentration in the medium to develop imazapyr tolerance in sugarcane. The stepwise approach has been used for the selection of salt (Queirós et al., 2007), herbicides e.g. glyphosate-tolerant plants (Nafziger et al. 1984) and heavy metals (Huang et al., 1987; Jackson et al., 1984) resistant variants. *D. Innoxia* cell lines resistant to cadmium (Cd) were obtained by a stepwise increase in Cd concentration (Jackson et al., 1984). A multi-step selection scheme requires more effort but in some cases, it is the sole means of selecting mutants from some cultures (Duncan and Widholm, 1990; Meredith, 1984). The stepwise exposure of cells to salinity stress is considered ineffective because a number of non-tolerant cells with a labile metabolism adapt to the gradual rise of salt over time (Ochatt et al., 1999; Queiros et al., 2007).

Table 3. *In vitro* screening and selection for resistance to mineral stress in plants

Trait screened or selected	Plant species	Explants	Culture system	Selection strategy	Trait stability	Plant Regeneration	Reference
Fe-efficiency	*P. amygdaliformis & C. oblonga*	shoot, leaf discs	callus, suspension	direct	stable cell lines	regenerated tolerant plants	Dolcet-Sanjua et al. (1992)
Fe-efficiency	*Prunus persica* rootstocks	intact plants	hydroponic culture	direct, recurrent	not tested	not tested	Gogorcena et al. (2004)
Fe-chlorosis resistance	*Pyrus pyraster Burgsd*	axillary buds, leaf, shoot apex	*in vitro* adventitious regeneration system	direct	stable tolerant lines	putative tolerant lines	Palombi et al. (2007)
Fe-deficiency tolerance	*Malus domestica*	shoots	micropro-pagation	direct	not tested	not tested	Mahdavikia and Mahna (2012)
Fe-efficiency	*Saccharum officinarum*	leaves	callus	direct	stable growth on Fe-stress medium	regenerated tolerant plants	Naik et al. (1990)
Ni tolerance	*Setaria italica*	leaf base, mesocotyl	callus	direct	stability maintained for prolonged period	tolerant plants regenerated	Rout et al. (1998)

Fe: iron; Ni: nickel

Table 3 continued.

Trait screened or selected	Plant species	Explants	Culture system	Selection strategy	Trait stability	Plant Regeneration	Reference
Cr and Ni tolerance	*Echinochloa colona*	leaf base, leaf tip, mesocotyl	callus	direct	stable tolerant plants	tolerant plants regenerated	Samantaray et al. (2001)
Pb, Zn & Fe tolerance	wetland plants	roots	hydroponic	direct	not tested	not tested	Denga et al. (2009)
Zn and Mn tolerant	*Brassica campestris, Brassica juncea*	cotyledon explants	callus	direct	tolerant calli maintained for a prolonged period	tolerant and non-tolerant regenerates	Rout et al. (1999)
Zn tolerance	*Setaria italica L.*	leaf base, mesocotyl	callus	direct	stable	tolerant plants regenerated	Samantaray et al. (1999)
Zn & Cu tolerance	*Agrostis stolonifera*	shoot tip meristem tissue, nodal root tip	callus	direct	tolerance to both traits maintained	Zn and Cu tolerant plants regenerated	Wu & Antonovics (1978)
Al-resistance	*Nicotiana plumbaginifolia*	petioles	plated cells, callus, cell suspensions	direct and rescue	Al resistance (mutant) stable	Al resistance trait passsed on to plant progeny	Conner and Meredith (1985)

Cr: chromium; Ni: nickel; Pb: lead; Zn: zinc; Mn: manganese; Cu: copper; Al: aluminium

Table 3 continued.

Trait screened or selected	Plant species	Explants	Culture system	Selection strategy	Trait stability	Plant Regeneration	Reference
Al-resistance	*Solanum tuberosum*	shoot segments	plated cells, callus, cell suspensions	gradual, direct	stable Al-tolerant clones	regenerates obtained	Wersuhn et al. (1994; 1988)
NaCl tolerance	*Solanum tuberosum*	leaf	callus	direct recurrent	stable salt-tolerant potato cell	salt tolerant plants regenerated	Ochatt et al. (1999)
NaCl tolerance	*Solanum tuberosum L*	leaf	callus	direct recurrent gradual	unstable beyond a year	not tested	Queirós et al. (2007)
NaCl tolerance	*Pennisetum purpureum schu (Napier grass)*	leaf	callus	direct and gradual	Short-term trait stability	plants regenerated	Chandler and Vasil (1984)
NaCl tolerance	*wheat cultivars*	immature embryos	callus	stepwise, direct	stable	plant regenerated	Barakat and Abdel-Latif (1996)
Cd resistant	*Lycopersicon peruvianum*	–	suspension	stepwise	resistance stable for 30 generations	not tested	Bennetzen and Adams (1984)
Cd resistance	*Datura innoxia*	–	suspension, protoplast	stepwise	stable after 400 generations	not tested	Jackson et al.(1984)

Al: aluminium; NaCl: sodium chloride

1.10 Justifications and aims of this research

Low bioavailability of Fe in soils is a major agricultural problem because inadequate Fe uptake results in chlorosis, reduction in crop quality and yields as well as a decrease in nutritional value of edible plant parts (Abadia et al., 2011; Hindt and Guerinot, 2012; Bert et al., 2013; García-Mina et al., 2013). *In vitro* selection of rare mutants or somaclonal variants with enhanced capacity to thrive on marginalised (iron deficient) soils and or efficiently utilise iron under such conditions is a considerably rapid and cost-effective means of combating IDC and maintaining the nutritional quality of crops. The identification and selection of Fe-efficient cell lines are essential for the enhancement of potato productivity especially in calcareous soils. The *in vitro* selection and use of Fe-efficient plants provide the best management approach for alleviating IDC and improving crop production (Zhu et al., 2015; Vasconcelos and Grusak 2014; Jelali et al., 2010). The development of Fe-efficient plants can complement strategies aiming to avoid excessive iron fertiliser application.

Potato, a staple diet for over a billion people daily, (King and Slavin, 2013; Barrell et al., 2013) delivers just 3-6% recommended daily allowance of iron to the body (Navarre et al., 2009; Lefevre et al., 2012). Potato is an ideal crop for *in vitro* selection of iron-efficient cell lines because iron can be made highly bioavailable owing to the low phytic acid and high vitamin C content in potato (Frossard et al., 2000; Love et al., 2004; Phillippy et al., 2004; Navarre et al., 2009; Haynes et al., 2012). The use of plant tissue culture to select for Fe-efficient potato plants has so far not been explored as an approach towards the development of new potatoes capable of iron biofortification. Efforts to maximise the efficient use of iron and achieve iron biofortification target levels in potatoes are in their elementary stages.

In vitro selection of iron-efficient potato variants can serve as a tool in establishing different lines to aid a better understanding of iron nutrition in potato. The current knowledge gap in mechanisms leading to iron acquisition and nutrition in potato appears to be a major impediment in devising approaches to improve its growth in marginal soils. It is therefore crucial that an understanding of the machinery and mechanisms controlling iron uptake and homeostasis in potato is developed using biochemical and molecular detection tools.

1.10.1 Objectives and thesis structure

The aim of this study was to investigate the effectiveness of *in vitro* plant tissue culture as a tool to obtain Fe-efficient potato cell lines and to characterise the selected lines. To achieve these, the specific objectives were to:

1. Select for Fe-efficient potato cell lines *in vitro* by exposing callus cultures to Fe-deficiency selective pressure using the direct selection strategy.

2. Regenerate plantlets on optimised regeneration medium and test the Fe-efficiency status of the callus-derived regenerants on growth regulator-free Fe-deficient medium.

3. Examine morphological and visual characteristics of potato callus cultures and plants in response to varying Fe supplies by measuring weight, growth area, shoot and root length, internodal distance, number of leaves per plant and visual rating of chlorosis development.

4. Investigate the biochemical responses of callus and plant cultures exposed to Fe-deficiency conditions and to distinguish between Fe-efficient and inefficient plant lines. Ferric chelate reductase activity, total phenolic, chlorophyll and carotenoid contents as well as antioxidant enzyme (peroxidase, catalase, and ascorbate peroxidase) activities are measured to evaluate the effect of iron deficiency stress conditions on these parameters in calli and plants. The cellular localisation of Fe and the detection of reactive oxygen species (H_2O_2) in calli were investigated using histochemical staining and light microscopy techniques.

5. Assess the relative expression levels of the iron-regulated transporter (*IRT1*) and Fe storage (ferritin: *FER*) genes associated with Fe acquisition and homeostasis. IRT1 and FER3 transcript levels were quantified to examine their expression patterns in Fe-efficient and Fe-inefficient plant lines. Plants regulate the levels of IRT1 in order to ensure the optimal absorption of iron. *IRT1* and ferritin expressions have been found to be influenced by iron deficiency at the transcriptional level (Barberon et al., 2011; Briat et al., 2010).

The thesis has been organised into seven chapters as described briefly below.

CHAPTER ONE

In this chapter a general introduction and an in depth literature review on all aspects of the study are provided. The importance and functions of iron in biological systems, causes and problems associated with Fe deficiency, mechanisms of iron uptake and homeostasis in plants and plant response to Fe-deficiency have been reviewed. In Section 1.7, the approaches to improving iron uptake and nutrition in plants are discussed. tissue culture-based selection of plants with desirable traits and the objectives of the study are outlined on Sections 1.8 and 1.11 respectively.

CHAPTER TWO

The details of the potato plant material, culture media and tissue culture growth conditions used in the study are provided in this Chapter. The experimental and biochemical analytical procedures are also described. Methodologies for RNA extraction, cDNA synthesis and the quantification of relative gene expression levels using RT-qPCR techniques are outlined in Section 2.7.

CHAPTER THREE

Plants evolve adaptive mechanisms under stress conditions. The study documents the chain of events that contribute to or that are associated with the development of responses in potato callus cells based on the Fe status of growth medium. In order to identify early and delayed responses to iron stress, potato calli after one and three months of Fe deficiency stress were examined to develop an understanding of morphological and biochemical response to Fe deficiency stress. Morphological and visual assessments of calli subjected to limiting Fe supplies for different culture durations are described and discussed in this Chapter. Short-term (one month) and long-term (three month) responses of calli under Fe deficiency conditions are reported. The set of analytical experiments designed to detect associations between different biochemical responses and duration of exposure to iron deficiency in potato calli are presented. Sampling of callus cultures and statistical analysis of data are described in Section 3.4. The results of biochemical responses of callus cultures to Fe-deficiency conditions as shown in Sections 3.6 -3.10 include: the concentrations of chlorophyll and

carotenoid, total phenolic content and enzyme (FCR, peroxidase, catalase, and ascorbate peroxidase) activities. Histochemical detection of ROS (hydrogen peroxide) and Fe localisation in callus cells is also presented.

CHAPTER FOUR

In vitro tissue culture conditions and cycle can induce the formation of somaclonal variants which can be exploited and harnessed for the selection and development of novel traits of agronomic impotence in plants. The callus culture system provides highly disorganised mass of cells that are easy to manipulate to obtain somaclonal variants. It is the best system to use in the case of this study in which regeneration of plantlets is desired. In this Chapter, the optimisation of *in vitro* culture conditions for callogenesis and plantlet regeneration based on use of varying concentrations of plant grow regulators is described and the results discussed. Preliminary experiments and studies on exposure of callus cultures to Fe stress (excess and deficient) conditions and investigation into the suitable selection strategies to employ are presented. The selection of somaclonal variants tolerant to Fe deficiency by the application of selective pressure and the regeneration of plantlets from isolated Fe-efficient calli lines are also described. Assessment of phenotypic variation among regenerants and micropropagation of the regenerants for the establishment of plant lines are discussed in Section 4.4.

CHAPTER FIVE

In this chapter the possibility of using morphological features and visual chlorosis scores as screening parameters to characterise plant lines as Fe-efficient or Fe-inefficient and to select *S. tuberosum* cv 'Iwa' lines tolerant to Fe deficiency-induced chlorosis is discussed. The post regeneration test performed to confirm the stability of Fe-efficiency trait in the established plant lines is described and discussed in Section 5.1.2. The results on the investigation into the morphological properties of the leaf, root and stem that might be associated with iron deficiency stress resistance are reported. It is necessary to decipher the biochemistry of tolerance and sensitivity to iron deficiency in order to biochemically characterise potato cell lines as EF or IFN. In Sections 5.5 -5.9, the results of various biochemical tests performed on the plant lines are presented and discussed with respect to the similarities and distinction between Fe-efficient plants and others. The biochemical reactions and responses that make

some plant lines more IDC tolerant are investigated to delineate the biochemical mechanisms associated with such a trait.

CHAPTER SIX

The investigations on gene expression responses relating to Fe transport and storage in potato plant lines with differential tolerance to Fe deficiency are reported in this Chapter. In Section 6.1, the details of the experimental design for gene expression analysis and data acquisition processes are described. Descriptions of RNA and DNA quality control tests and the optimisation of qPCR assay are given in Section 6.2. The expression of two key iron deficiency-related genes, the iron-regulated transporter, *irt1*, and ferritin (*fer3),* was assessed using RT-qPCR to detect and quantify mRNA levels. The results and discussion on the transcriptional patterns of *irt1* and *fer3* genes in plants lines exposed to Fe-deficiency are presented in Sections 6.3 and 6.4.

CHAPTER SEVEN

The main conclusions of the study and recommendations for future research are provided in this Chapter. The key findings discussed in Chapters three to six are summarised together with their implications, possible applications of the *in vitro* selection scheme designed used.

CHAPTER TWO

General materials and methods

2.0 Tissue culture working environment

Tissue culture is very delicate and highly sensitive to contamination. To prevent contamination, all experiments were carried out under aseptic conditions. All aseptic manipulations were done inside a laminar flow cabinet which was turned on for at least 30 minutes before use to ensure constant sterile air flow within the work area. Additionally, all working surfaces within the laminar flow cabinet were cleaned with cotton wool saturated with 70% (v/v) ethanol before the laminar flow cabinet was used. Ethanol (70 or 100%) was used on all other items (gloves, forceps, scalpels, containers) used for culturing plants in the laminar flow cabinet. Forceps and scalpels were placed in heated (300-350^{o}C) glass bead steriliser for 10-15 min and allowed to cool before use.

2.1 Plant material and explant preparation

The potato plant, *Solanum tuberosum* L. (cv. 'Iwa'), used in this study was obtained from aseptically grown potato plantlets (stock plants) prepared at the Plant Biotechnology Laboratory, University of Canterbury, New Zealand (Yoon and Leung, 2004). The 'Iwa' cultivar was eventuated from a cross between Tahi and an Australian virus-resistant line (Genet, 1985). 'Iwa' is superior to Ilam Hardy and Rua at maturity and is the third most important cultivar in New Zealand behind Rua and 'Ilam Hardy' (Genet, 1985). The 'Iwa' potato is a model plant; it has good field resistance to early and late blight as well as virus X and Y (Genet, 1985). The main advantages in using *Solanum tuberosum* as a model plant system include being easy to grow, easy to manipulate during *in vitro* culture and the extensive studies that have been conducted in potato plant physiology and genetics.

The type of explants used for callogenesis was carefully chosen since various parts of the potato plant respond differently under *in vitro* conditions in terms of variant production and regeneration efficiency. Young leaf explants were used in this study because 'Iwa' potato leaf tissue was the most responsive tissue for callus induction (David Leung, personal communication, August 2013). Young leaf explants for *in vitro* culture were excised from the leaf stalk (petiolule region) with sterile scalpel and forceps under sterile conditions in a

laminar flow transfer hood. Nodal explants were used for micropropagation of the potato plants.

2.2 Culture media

All tissue culture media used for all experiments were prepared based on MS (Murashige and Skoog, 1962) medium. Iron was mainly supplied in the form, FeNaEDTA. Stock solutions for basal MS medium were prepared as follows (see Appendix A1); macronutrients (10x), micronutrients (100x), organic supplements (100x) and FeNaEDTA (100x) and stored at 4˚C for 3 months or longer at -20˚C. MS medium was freshly prepared by adding the required amounts of stock solutions, 3% w/v sucrose (AppliChem, GmbH, Germany) and MilliQ water. New stock solutions were prepared every 3-4 months to ensure that the media were always of good quality. A half-strength MS medium (was used with the following nutrient concentrations (mg L^{-1}); 825 NH_4NO_3, 950 KNO_3, 85 KH_2PO_4, 220 $CaCI_2.2H_20$, 185 $MgSO_4.7H20$, 0.415 KI, 3.1 H_3BO_3, 0.125 $Na_2MoO_4.2H_20$, 0.0125 $COCl_2.6H_20$, 11.15 $MnSO_4.4H_20$, 4.3 $ZnSO_4.7H_2O$, 0.0125 $CuSO_4.5H_20$, 18.7 $Na_2EDTA.2H_20$, 13.9 (50 µM) $FeSO_4.7H_20$, 0.05 thiamine-HCI, 0.25 pyridoxine-HCI, 0.25 nicotinic acid, 1.0 glycine, 50 myo-inositol.

All media used in this study were adjusted to pH 5.7-5.8 with a pH meter using either 0.1 M NaOH (to increase pH) or 0.1 M HCl (to decrease pH) and subsequently distributed into glass bottles or dispensed (30-40 ml) into polycarbonate tissue culture vessels. Media were gelled with 0.8% (w/v) agar (Agar technical No. 3, Oxoid Ltd, UK) and autoclaved (wet heat sterilisation) for 20 min at 121˚C. After autoclaving, media in glass bottles were poured (about 25-30 ml) into sterile plastic Petri dishes (9 cm in diameter), allowed to cool for 30 mins and sealed with parafilm. Autoclaved media were stored overnight in the dark to check that there was no visible microbial growth.

2.2.1 Callus induction medium (CIM)

Media for callus induction (CIM) were comprised of half-strength MS medium supplemented with α-napthaleneacetic acid (NAA) and N_6-benzylaminopurine (BA). Plant growth regulators (PGRs) were added prior to pH adjustment (5.7-5.8) and the addition of agar (0.8% w/v). PGRs were added to the MS medium to make up different combinations and concentrations of BA (0, 0.89, 1.78, 2.66, 3.55, 4.44, 6.66, and 8.88 µM) and/ or NAA (0, 1.07, 2.15, 3.22, 4.30, 5.37, 8.05, 10.7 µM). Thirty-three media formulations (see Appendix

A: Table A3) were used to test for the development of primary callus. Experiments on callus proliferation (using primary callus) and induction (from young leaf explants) on optimal CIM of varying FeNaEDTA (see Appendix, Table A4) concentrations (0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 400 and 800 µM) were performed. Na_2EDTA in all media was adjusted to the EDTA concentration (50 µM) as in the control, 50 µM FeNaEDTA medium.

2.2.2 Selection medium (SM)

Constituents of media for the screening and selection of Fe-efficient somaclonal variants were half-strength MS of different FeNaEDTA concentrations supplemented with 3.22 µM NAA and 1.78 µM BA. FeNaEDTA concentrations of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 µM (see Appendix, Table A4) were used as Fe deficiency selective pressure and 50 µM as control (sufficient or original FeNaEDTA concentration in half-strength MS medium).

2.2.3 Regeneration medium (RM)

RM was composed of half-strength MS medium of different concentrations and/or combinations of the PGRs, NAA, BA and gibberellic acid (GA_3). Thirty-two different media preparations (see Table 8) were used to test for the regeneration potential of callus cultures. MS medium supplemented either NAA only or without PGR served as negative controls. It was assumed that the cytokinin, BA, and GA_3 either solely or in combination could cause callus to regenerate into plants. This assumption was based on the fact that cytokinins stimulate axillary and adventitious shoot proliferation.

2.3 Clonal propagation of stock plants

Micropropagation of aseptically cultured stock plants was carried out on half-strength MS medium to obtained ample plant material for all experiments. Apical sections were used since they are most likely to produce plants that are true to type. The explants were excised just below the second node from the apical region of the stem. The length of the explants was about 10-15 mm. Four nodal explants of *in vitro* potato plantlets (stock plants) provided were cultured in a 40 ml half-strength MS medium in 250 ml clear polycarbonate tissue culture vessel. Plants were maintained in a growth room under sterile and controlled environmental conditions at 22 ±2 ^{o}C, with 24 hr photoperiod (cool white lighting). Plants were multiplied

every four weeks by subculturing into fresh half-strength MS medium. Many clonal plants were obtained and subsequently used for callus induction and selection experiments.

2.4 Callus induction

Callus induction responses from leaf explants on 33 different callus induction medium formulations (see Table 4) were investigated to ascertain and develop the most favourable culture medium for inducing callus and sustaining its growth. Young leaf explants from one month old clonally propagated stock plants were aseptically excised using sterilised scalpel and forceps and transferred to an autoclaved CIM in a sterile Petri dish. Four leaf explants were cultured per Petri dish containing 20-25 ml of CIM. The explants were gently pressed onto the agar-solidified medium to ensure good contact. Petri dishes were sealed with parafilm to prevent desiccation. The experiment was carried out with 12 biological replicates (four explants each in three Petri dishes) repeated three times using varying concentrations and combinations of NAA and BA (see Appendix, Table A3) in preliminary studies. The agar plates were placed horizontally inside a temperature controlled room under constant illumination (24 hr photoperiod) at 22 ± 2 ˚C in a completely randomised fashion for 4 weeks. Callus cultures were monitored for primary callus production after 7 and 21 days of inoculation. The data were expressed as percentage of explants producing callus. Concentrations of the supplemented PGRs (3.22 µM of NAA and 1.78 µM of BA) in half-strength MS medium found to be optimal for callus induction were used for all further callus initiation and growth experiments. Experiments on callus induction from leaf explants on CIM with varying Fe concentrations (0-800 µM) were also conducted.

2.4.1 Callus proliferation

After 4 weeks, primary callus which had developed at the cut edges of the leaf explants were subcultured onto CIM of corresponding FeNaEDTA concentration. The callus cultures were proliferated and maintained on the same medium by regular subcultures at 4-weeks intervals in a temperature controlled room at 22 ± 2^{o}C under constant illumination (24 hr photoperiod). Further experiments involving subculturing callus on media of varying Fe supplies were carried out.

2.5 Plantlet regeneration from potato callus cultures

2.5.1 Shoot formation

To investigate the effect of PGRs on plant regeneration from callus cultures and identify an optimal regeneration culture medium, calli formed on optimal CIM were subcultured on 32 formulations of regeneration medium (see Table 8). Four excised pieces of calli (0.2-0.3 g) were cultured on 35-40 ml RM in 250 ml polycarbonate tissue culture vessels and placed under continuous cool white fluorescent light at 22 ±2^{o}C. To induce the generation of shoot buds, calli were transferred at 4-week intervals to fresh RM of corresponding composition. The experiment was repeated four times. The RM (half-strength MS supplemented with 6.66 µM BA and 2.89 µM GA_3, (B5G5) that favoured the development of shoot buds and plantlets was used for culturing calli isolated from the selection media (see Figure 5).

2.5.2 Shoot proliferation and induction of rooting

Calli which developed shoot buds were transferred to half-strength MS medium devoid of PGRs for plant propagation. All the cultures were grown in 250 ml polycarbonate tissue culture vessels incubated in a 22 ±2°C temperature controlled room under continuous cool, white fluorescent lamps for 4 weeks. The calli were subcultured either in full or in part in half-strength PGR-free MS medium. Individual adventitious shoots of 1.5 cm or taller, were carefully excised, devoid of callus, to prevent carry-over effects. Subsequently, 3-4 subcultures onto PGR-free half-strength MS medium were required for shoot elongation and rooting (Figure 5). The plantlets were subcultured at one month intervals. Shoots developed from the selected Fe-efficient callus lines were micropropagated on half-strength MS medium. Micropropagation of plantlets was carried out for further two to three subcultures (3-4 months) to achieve sufficient proliferation of regenerants.

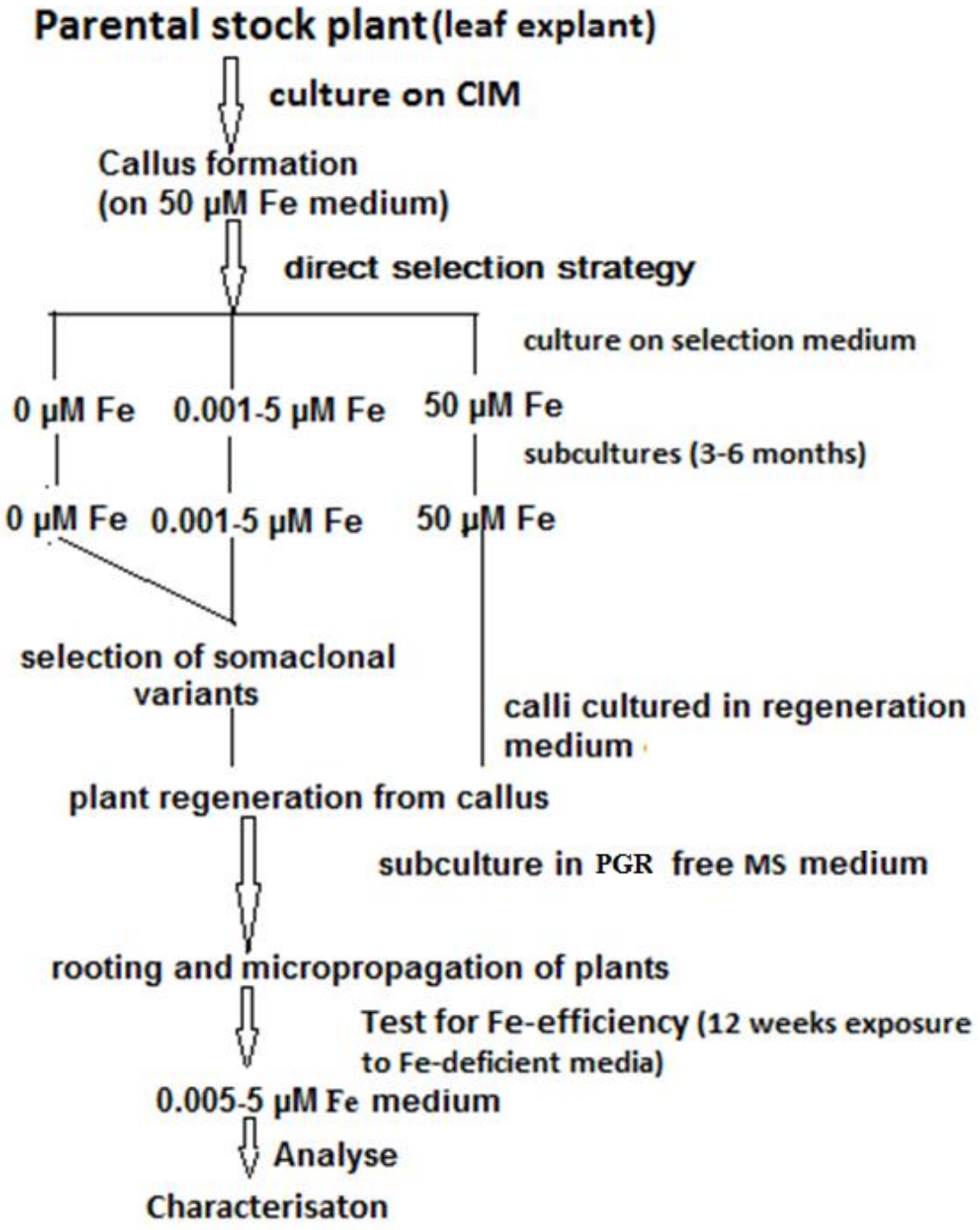


Figure 5. Scheme for the *in vitro* selection of potato variants for their tolerance to Fe-deficiency.

2.6 Biochemical Analysis

2.6.1 Ferric chelate reductase (FCR) activity

FCR activity was determined using the methods described by Robinson et al. (1997) and Ihemere et al. (2012) with some modifications. The formation of the Fe (II)–bathophenantroline disulfonate complex from Fe (III)-EDTA and bathophenantroline disulfonic disodium salt (BPDS) was estimated spectrophotometrically. Prior to assay, callus and intact root samples were washed in 1 mM Na_2EDTA for 5-10 min to eliminate apoplastic Fe and/or extra Fe in attached gelled medium followed by three times wash with MiliQ water to reduce excess EDTA (Kabir et al. 2015). This was done to prevent FeNaEDTA content in medium interfering with the analysis. To avoid any reduction due to ultraviolet light, solutions were freshly prepared and the containers wrapped in aluminium foil for the test period. Elicitation of FCR activity was investigated in an assay medium comprising 1× MS medium (without Fe), 5 mM MES (2-(4-morpholino) ethanesulfonic acid) of pH 5, 0.1 mM Fe (III)-EDTA and 0.3 mM sodium bathophenanthroline disulfonic acid (BPDS). FCR activity in intact roots and leaves was investigated by placing samples in 1 ml assay medium (see Appendix B.1). Samples were incubated in the reaction media in Eppendorf tubes under constant shaking for 30 min in the dark. The absorbance of the supernatant was then measured at 535 nm to evaluate the concentration of the Fe (II)–BPDS complex. Next, samples were blotted dry on Whatman filter paper and weighed.

Callus samples were weighed (about 150 mg) into1.2 ml assay medium and homogenised on ice using a mortar and pestle. The homogenate was then poured into an Eppendorf tube and kept on shaking on a shaker with a constant speed of 70 rpm in the dark at ambient temperature (Deshattiwar et al., 2009). After 30 min, the samples were centrifuged (Eppendorf 5810R Centrifuge) at 10 000 g for 10 min at 4^{o}C in order to obtain a clear supernatant free of callus material. To estimate the concentration of the Fe (II)–BPDS complex, the absorbance of the supernatant (1 ml) was measured at 535 nm.

Blank measurements (without samples) were made to correct for any non-specific Fe reduction. The rate of ferric reduction was calculated as μmol Fe (II)-BPDS per gram fresh weight per hour. The molar extinction coefficient used was 221,401 $M^{-1}cm^{-1}$ (Bruggemann et al., 1993).

2.6.1.1 Modifications to disruption of callus cultures for FCR assay

Due to the hard and compact nature of the calli samples, some modifications were made to ensure effective and or/ optimal interaction between sample and reaction medium. Various ways of disrupting/loosening the cells to ensure maximal interaction with the assay medium were employed as outlined below;

I. use of scalpels with stainless steel blades to excise calli placed in assay medium in an Eppendorf tube
II. use of plastic forceps to break up calli while in contact with assay medium in an Eppendorf tube
III. Samples were weighed (about 150 mg) into1.2 ml assay medium and homogenised with mortar and pestle on ice. The homogenate was then poured into an Eppendorf tube.

Alternative III was chosen to be most effective in the breaking up the calli into a mixture of fine cells. This is because it was impossible to attain the same degree of calli breaking up the calli across all samples by using either the blade or plastic forceps. Furthermore, stainless steel blades appeared to react with the assay medium since the blank mostly turned light pink when the blade was immersed in. It was therefore assumed that interaction of the blade with the assay medium may have produced a background effect that interfered with the absorbance readings.

2.6.2 Chlorophyll and carotenoid determination

Chlorophyll and carotenoid content were quantified according to the method of Arnon (1949). Harvested callus, plant leaf and root samples were weighed (100 mg) and homogenised in 1.5 ml of 80% acetone on ice. Gelled nutrient medium was removed from roots by washing roots thoroughly with distilled water and then quickly blotted in tissue paper prior to weighing and acetone extraction. The homogenate was poured into an Eppendorf tube and centrifuged at 10 000 g for 5 min at 4^{o}C. The supernatant (extract) was dispensed into a cuvette and the absorbance readings at 645, 663 and 470 nm were recorded. Calculations for chlorophyll concentrations were made based on absorbance readings at wavelengths, 645 and 663 nm (Wellburn and Lichtenthaler, 1984; Duxbury and Yentsch 1956). Pigment content was expressed as mg/ml.

The chlorophyll and carotenoid contents were calculated as follows;

Chlorophyll a (mg/ml) = 12.7 A663 - 2.69 A645 ……. (2)

Chlorophyll b (mg/ml) = 22.9 A645 - 4.68 A663 ……. (3)

where:

A645 = absorbance at a wavelength of 645 nm

A663 = absorbance at a wavelength of 663 nm

A470 = absorbance at a wavelength of 470 nm

Total Chlorophyll (mg/ml) = Chlorophyll a + Chlorophyll b.

Carotenoid (mg/ml) = $\frac{\mathbf{1000}\boldsymbol{A}\mathbf{470}-\mathbf{3.27}[\boldsymbol{chla}]-\mathbf{104}[\boldsymbol{chlb}]}{\mathbf{229}}$ ……. (4)

2.6.3 Measurement of total phenolics

Total phenolic content in calli were evaluated using Folin-Ciocalteu (F-C) reagent according to the method described by Spanos and Wrolstad (1990). Phenolics react with phosphomolybdic-phosphotungstic acid reagent like the F-C reagent to produce a blue colouration that can be measured as absorbance at 765 nm. Freshly weighed (100 mg) callus, leaf and root samples were ground on ice in 1.5 ml acetone (80% v/v) using a mortar and pestle. Roots were washed thoroughly with distilled water to remove bound agar medium and then quickly blotted in tissue paper prior to the analysis. The homogenate was then centrifuged at 10 000g for 5 min at 4^{o}C. Acetone extracts of the samples were used to perform the assay. The reaction mixture contained 50 µl of sample acetone extract, 450 µl H_2O, 2 ml of 7.5% (w/v) Na_2CO_3 and 2.5 ml freshly diluted (1/10 dilution) F-C reagent. The reaction mixture in glass tubes with screw tops were vortexed (10 s) to ensure that all components of the assay were well mixed. The tubes were incubated (water bath) at 45^{o}C for 15 min, allowed to cool to room temperature and the absorbance read at 765 nm. Blank controls contained the reaction mixture without sample (mixture + 80% v/v acetone). Assays were carried out on duplicate extracts per sample and duplicate assays per extract. The calibration curve of Gallic acid was prepared using different dilutions of stock solution (1 mg/ml) freshly prepared in 80% acetone. Gallic acid concentrations of 0, 4, 8, 12, 16 and 20 µg/µl were used for development of the standard curve (see Appendix Table B1). Phenolic assay was then performed by the addition of 2 ml 7.5% (w/v) Na_2CO_3 and 2.5 ml freshly diluted (1/10 dilution) F-C reagent as done for the plant samples. Total phenolic content was determined using a standard curve prepared with Gallic acid and the results expressed as milligram Gallic acid equivalents per gram of tissue (mg GAE/g).

2.6.4 Lipid peroxidation (TBARS contents)

The Thiobarbituric Acid Reactive Substances (TBARS) assay is a direct quantitative measurement of malondialdehyde (MDA), a biomarker of lipid peroxidation. The deleterious effect of ROS production on cell integrity was estimated by measuring MDA levels in calli. The analysis was carried out using an adaptation of methods by Demiral and Türkan (2005) and Salama et al. (2009)**.** About 300 mg of fresh calli (of similar culture age) were ground in liquid nitrogen using a pestle and mortar and then homogenised in 3 ml of ice-cold 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (Eppendorf 5810R Centrifuge) at 12,000 g for 20 min at 4°C. Next, 1 ml aliquot of the supernatant was mixed with 2 ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) and heated at 95°C for 30 min. For the blank, 1 ml of the supernatant was added to 20% TCA without TBA. Subsequently, the reaction product was quickly cooled on ice and centrifuged at 12,000 g for 15 min at 4°C. High temperatures accelerate the reaction and low temperatures inhibit it. Since TBA can interact with MDA and produce a red compound in acidic buffer, MDA content can be calculated by estimating the amount of the red compound. Lastly, the absorbance of the supernatant was read at 532 nm and then it was corrected by subtracting the absorbance at 600 nm. The concentration of the MDA-TBA complex produced was calculated using the molar extinction coefficient, ε =155 mM^{-1} cm^{-1}. The level of lipid peroxidation was expressed as μmol MDA g^{-1} FW. Two extractions were made and two assays were carried out per extract.

2.6.5 Antioxidant activity assays

2.6.5.1 Guaiacol peroxidase assay

Peroxidase activity was determined by measuring the rate of guaiacol oxidation at 470 nm Ranieri et al. (1997). Frozen callus samples (50 mg) collected at 4 and 12-week intervals were homogenised with a mortar and pestle in cold 0.1 M potassium phosphate buffer (pH 6.6). Freshly weighed (50 mg) plant leaves and roots were flash frozen in liquid nitrogen and stored at -80 ^{o}C prior to homogenisation in cold 0.1 M potassium phosphate buffer (pH 6.6). Total intact roots developed by each plant sample were first washed three times with MiliQ water to remove nutrient and then quickly blotted in tissue paper. The homogenate was centrifuged at 10 000g for 5 min at 4°C and the supernatant (crude enzyme extract) was used for the peroxidase assay.

The reaction mixture (1 ml) composed of 0.1M phosphate buffer (pH 6.6), 5 µl of 10% H_2O_2, 16 µM guaiacol substrate (Sigma, St Louis, USA) and 100 µl (10x diluted) of crude enzyme extract. The blank control was made up of the assay mixture without the enzyme. The formation of tetraguaiacol was observed as a brown colouration. Absorbance was measured at 470 nm with a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) after 2 min incubation at room temperature. The reaction was followed at 10 s intervals for 120 s to give a linear rate of tetraguaiacol formation. Peroxidase activity was expressed as absorbance units per min per gram fresh weight. Assays on plant organs were carried out on duplicate extracts per sample and duplicate assays per extract. Three calli extracts were prepared per treatment (representing 9 biological replicates from 3 Petri dishes) and two assays were performed per extract.

2.6.5.2 Catalase (CAT) and ascorbate peroxidase (APX) enzyme assays

Catalase and ascorbate peroxidase (APX) activities were determined according to methods used by Aebi (1984) and Ramírez et al. (2013).

a) Sample preparation and enzyme extraction

Frozen calli (0.5 g) were ground into a powdered form in a mortar and pestle with liquid nitrogen. Soluble proteins were extracted by homogenisation of the powdered samples in 1.5 ml chilled 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpolypyrrolidone (PVP-40) and 1 mM phenylmethylsulfonylfluoride (PMSF). The extraction buffer (see Appendix B7) for APX activity additionally contained 5 mM ascorbate. The homogenates were centrifuged (Eppendorf 5810R Centrifuge) at 20,000 g for 20 min at 2°C. The supernatant (crude extract) was stored at -80 ºC for further analysis of protein concentration and enzyme activity.

b) Total protein assay

Using bovine serum albumin (BSA) as a standard, protein concentration was determined according to Bradford (1976). The calibration curve of BSA was prepared using different dilutions of a BSA stock solution (1 mg/ml). A range (0-100 µg/ml) of BSA solutions was used as the standard. The enzyme extract (100 µl) was mixed (by vortexing) with 1 ml of Bradford reagent to make up 1.1 ml and incubated for up to 5 min. The absorbance was then measured at 595 nm. A reagent blank was prepared from 100 µl of the protein preparation buffer (50 mM phosphate buffer of pH 7.0) and 1 ml of Bradford reagent. Protein

concentration of samples was determined using a standard curve prepared with BSA and the results expressed as µg/ml.

c) Ascorbate peroxidase (APX) Assay

A reaction mixture consisting of 50 mM phosphate buffer (pH 7.0), 0.2 mM H_2O_2, 0.5 mM ascorbate and 50 µl of enzyme extract in a final volume of 1 ml was used for the determination of APX activity. APX activity was measured by following the oxidation rate of ascorbate continuously at 290 nm for 2 min using on a UV-Vis spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) to obtain a linear rate of reaction. The concentration of oxidised ascorbate was calculated using extinction coefficient ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of APX was defined as the amount of enzyme oxidising 1.0 µmol of ascorbate per min. APX activity was expressed as nmol of $H_2O_2\ min^{-1}\ mg^{-1}$ protein.

d) Catalase (CAT) Assay

CAT activity was measured in a reaction solution containing 10 mM H_2O_2, 50 mM phosphate buffer (pH 7.0) and 50 µl of enzyme extract in a final volume of 1 ml in a quartz cuvette. CAT activity was determined by monitoring the consumption of H_2O_2 at 240 nm. The reaction was followed on a UV-Vis spectrophotometer continuously for 2 min to give a linear rate of decomposition. One unit of CAT was defined as the amount of enzyme dismuting 1.0 µmol of $H_2O_2\ min^{-1}$. CAT activity was presented as nmol H_2O_2 decomposed $min^{-1}\ mg^{-1}$ protein using $\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$.

2.6.6 Histochemical detection of iron using the Perls stain

The *in situ* Perls staining technique which involves staining Fe on histological sections was performed as described by Roschzttardtz et al. (2009, 2013). Callus samples were initially rinsed with 1mM NaEDTA solution (for 5-10 min) followed by three times washing in MiliQ water to remove traces of nutrient medium and agar. The calli were then fixed with a fixation solution (see Appendix B6) containing paraformaldehyde (2% w/v), glutaraldehyde (1% v/v), caffeine (1% w/v) in 0.1M phosphate buffer (pH 7.0) for 30 min and incubated for 15 hr in the same solution. This was followed by washing (three times) with 0.1M Na-phosphate buffer (pH 7.4). The fixed samples were then dehydrated through a stepwise dehydration in a graded series of 50, 70, 90 and 100% ethanol, butanol/ethanol 1:1 (v/v) and 100% butanol for approximately 45 min in each. After clearing in toluene for 4 hours, tissues were infiltrated in

Paraplast (Plain) wax overnight, with repeated cycling vacuum, in a Shandon Citadel Autoembedding Machine (Thermo Scientific Shandon Citadel 1000 Tissue Processor). Tissues were later blocked in Simport moulds on a Tissuetek Blocking Station and the Paraplast blocks were sectioned on a Leica 2165 Rotary Microtome at 7 µm. Ribbons were floated onto slides (smeared with Haupt's Adhesive) and warmed on a hotplate to expand and flatten the sections. Finally, slides were drained and stored at 37ºC to dry and coagulate the adhesive, for at least 12 hours.

The *in situ* Perls staining procedure was carried out on sectioned tissues deposited on glass slides. Some slides were stained without dewaxing, to compare against dewaxed slides. Dewaxing was achieved by immersing the slides in warm xylene (45-50ºC) for 3 min or at room temperature for 5 minutes. Next, dewaxed slides were rehydrated through successive baths in xylene/100% ethanol (1:1 v/v), decreasing concentrations of ethanol (100, 90, 70 and 50%) and distilled water for 3 min each prior to staining. Slides were subsequently stained, ensuring that the sections did not dry out at any stage once dewaxed. Slides of dewaxed tissues placed in a transfer rack and vacuum infiltrated with equal volumes of 4% (v/v) HC1 and 4% (w/v) K-ferrocyanide (Perls stain solution) for 15 min and incubated for 30 min at room temperature. Following the Perls staining, samples were washed (at least 3x) with distilled water and incubated (for 1hr) in a methanolic solution containing 0.01M NaN_3 and 0.3% (v/v) H_2O_2. Next, the samples were washed with 0.1M phosphate buffer (pH 7.4). After staining, all slides were dehydrated in successive baths in distilled water and 50, 70, 90 and 100% ethanol for 3 min each, cleared in xylene for 3 min, and mounted in Eukitt Mounting Medium with coverslips. Slides were then dried in a 37ºC oven for 2 days and studied using light microscopy (Nikon Eclipse 80i and Leica TCS SP5).

2.6.7 Histochemical detection of hydrogen peroxide

The *in situ* detection of hydrogen peroxide was carried out by staining callus samples with 3,3'-diaminobenzidine (DAB) as described by Daudi et al. (2012) and Ramírez et al. (2013). For H_2O_2 detection, thin hand sections of calli were incubated in a 2 ml DAB staining solution in bijoux bottles for 18 hr (overnight). The staining solution contained 1 mg/ml of DAB in HCl (pH 3.6). The bijoux bottles were covered with aluminium foil (since DAB is light-sensitive). The control treatment was placed in 2 ml of HCl solution (pH 3.6) without DAB. The foil was removed following incubation and the samples were transferred into glass tubes. The DAB staining solution was replaced with a bleaching solution (ethanol: acetic

acid: glycerol; 3:1:1) and the tubes were carefully placed in a boiling water bath (~90-95 °C) for 15 ± 5 min. Chlorophyll content was bleached out leaving the brown precipitate formed by the DAB reacting with the hydrogen peroxide. The callus samples were thereafter placed in fresh bleaching solution and allowed to stand for 30 min. Samples could be stored at 4 °C for up to 4 days with no detrimental effects. The samples were visualised using a stereomicroscope (Leica MDG33, Leica Microsystems). The presence of H_2O_2 was indicated by the formation of a brown precipitate produced by the oxidation of DAB due to the presence of H_2O_2.

2.7 Reverse transcription-quantitative PCR (RT-qPCR) analysis

Gene expression studies were performed based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009; Huggett et al., 2013).

2.7.1 Nucleic acid sample preparation

2.7.1.1 Sample handling and working conditions

Tissue sampling, RNA extraction and storage influence the ability to obtain high-quality RNA that is free of RNase contamination (Fleige and Pfaffl, 2006). Therefore, liquid nitrogen was used to flash freeze samples immediately after harvesting and for grinding samples for RNA extraction. All samples were stored at -80^{o}C prior to and after RNA extraction in order to preserve full-length RNA. RNA is inherently susceptible to RNases, which are extremely stable and active therefore, care was taken to eliminate RNAse contamination in order to maintain the RNA quality and integrity. RNA processing was carried out under RNase-free working conditions to ensure that RNA was kept intact. Gloves were worn at all times and frequently wiped with RNaseZap (Sigma-Aldrich, St. Louis, USA). RNA isolation was carried out in a Class 2 Safety Cabinet which was initially sterilised using UV radiation and wiped with 70% ethanol and RNaseZap. The surfaces of the working area, pipettors, plastic ware and other RNA extraction equipment were wiped with RNaseZap. RNase-free aerosol filter barrier pipette tips as well as autoclaved mortar and pestle and microcentrifuge tubes were used.

2.7.1.2 RNA isolation

RNA extraction was carried using the RNeasy Plant Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for isolating total RNA from plants. The RNA was treated with RNase-free DNase I (Qiagen GmbH, Germany) to eliminate genomic DNA contamination that may produce false positive amplification signals.

First, 100 mg of root or leaf sample was weighed into a liquid nitrogen cooled pre-sterilised mortar and ground thoroughly in liquid nitrogen using a sterilised pestle. The powder obtained was transferred into a 2 ml RNase-free microcentrifuge tube previously cooled in liquid nitrogen. Care was taken throughout to avoid thawing of the sample. Exactly, 450 µl of reconstituted RLT lysis buffer (10µl β-mercaptoethanol to 1ml RLC) was immediately added to the sample and vortexed vigorously for about 1-2 min. Using a 1ml cut-through pipette tip, the lysate was transferred directly onto the QIA shredder spin column (in a 2 ml collection tube). Centrifugation was then carried out at 13000 rpm for 2 min. Next, the supernatant was carefully transferred into a new microcentrifuge tube without disturbing the pellet. To the cleared lysate, 0.5 vol of 100% RNase-free ethanol was added and mixed quickly by pipetting. The mixture (650 µl) was then applied together with precipitates formed to the RNeasy mini column. With the tube firmly closed, centrifugation was done at 13000 rpm for 15 s after which the flow throw was discarded.

RWI buffer (350 µl) was added to the spin column and incubated for 5 min at room temperature. To eliminate possible contamination of gDNA, DNase digestion was performed on-column using an RNase-free DNase I according to the manufacturer's instructions. After centrifugation at 13000 rpm for 15 s, the flow-through was discarded and 10 µl of DNase I stock solution was added to buffer RDD. Mixing was performed gently by inverting the tube since DNase I is sensitive to physical denaturation. The mixture was centrifuged briefly to collect residual liquid. DNase I incubation mix (80 µl) was added directly to the RNeasy spin column membrane and incubated at room temperature for 15 min.

Next, 350 µl Buffer RW1 was added to the spin column, centrifuged at 13000 rpm for 15 s and the flow-through was discarded without contacting the spin column. Centrifugation was repeated at 13000 rpm for 15 s. A volume of 500 µl of buffer RPE (reconstituted by the addition of 4 vols of 100% RNase-free ethanol) was pipetted onto the RNeasy column. This was followed immediately by centrifugation at 13000 rpm for 15 s. Subsequently, additional 500µl of RPE buffer was pipetted onto the RNeasy column and centrifuged at 13000 rpm for 3 min to dry the RNeasy silica-gel membrane. The RNeasy column was placed in a new 2 ml collection tube and centrifuged for 1 min at 13000 rpm. To elute the RNA, the RNeasy

column was transferred into a new 1.5 ml collection tube. Finally, a volume of 30 – 60 µl RNAse-free water was added directly onto the RNeasy silica-gel membrane, incubated at room temperature for 5 min and the tube centrifuged for 1 min at 13000rpm. The RNA elute was aliquoted (5 -10 µl) into sterile 0.5 ml Eppendorf tubes and stored at -80ºC for further analysis.

2.7.1.2 cDNA synthesis

Complementary DNA (cDNA) was synthesised from RNA by reverse transcription according to the instructions for Transcriptor Reverse Transcriptase (Roche Diagnostics GmbH, Penzberg, Germany). DNase/RNase-free consumables were used for all procedures. Samples and reagents were placed on ice and allowed to thaw prior to starting the reaction. Equivalent RNA quantities and comparable reaction conditions were used in all experiments in order to minimise variations in cDNA synthesis to ensure reliability of the results. Initially, denaturation of RNA secondary structure was carried out by incubating 1 µg RNA, 1 µl Oligo-p$(dT)_{18}$ primer (50 pmol), 1 µl random hexamer primer p$(dN)_6$ (100 pmol), 0.5 µl RNA secure (25x) and DEPC-H_2O in a total volume of 11 µl at 65ºC for 10 min in a thermocycler. Next, the mixture was cooled for 5 min on ice. The following were then added to each mixture; 9 µl master mix composed of 4 µl Expand reverse transcriptase buffer (5x), 2 µl DTT (100 mM), 2 µl dNTP-mix (10 mM each) and 1µl transcriptor reverse transcriptase (50 U/µl). The 20 µl reaction mixture was then incubated at 25ºC for 10 min and subsequently at 42ºC for 60 min. The enzyme was inactivated at 70ºC for 15 min. The synthesised cDNA was diluted 10-fold with TE buffer and stored at -80ºC for further analysis. The reverse transcription of RNA was carried out in duplicates per sample. A negative control (RNA sample without reverse transcriptase) used to detect gDNA contamination was also prepared (Nolan et al., 2006).

2.7.2 Quality and quantity of nucleic acids

2.7.2.1 Absorbance

The quantity and quality of RNA or DNA were assessed using NanoDrop ND-1000 spectrophotometer V3.2 (BioLab Nanodrop Technologies, USA). Nucleic acid concentration was determined by pipetting 2 µl of RNA/DNA extract on to the pedestal of the NanoDrop spectrophotometer after blanking the instrument with 2 µl of RNase-free water. RNA purity

was estimated by the absorbance at 260, 280 and 230 nm (A_{260}/A_{280} and A_{260}/A_{230}). The A_{260}/A_{280} ratio was measured in Tris buffer at neutral pH. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10 mM Tris at pH 7.5. RNA concentration (in ng/µl) was measured at 260 nm. Quantification of RNA ensured that similar amounts of total RNA were used in all reverse transcription reactions from different tissue samples.

2.7.2.2 Agarose gel analysis

Using gel electrophoresis technique, the integrity of the extracted RNA was assessed by visualisation of ribosomal bands on agarose gel using Mini Sub DNA Cell apparatus (BioRad laboratories Inc. USA). For a 1% agarose gel, 0.3 g agarose (UltraPure Agarose, Invitrogen) was weighed into 30 ml 1 x TAE buffer (see Appendix C1) and heated in a microwave oven until completely melted. After the gel solution has cooled down to about 60°C, 3 µl of SYBR Safe gel stain (Invitrogen, USA) was added and mixed by swirling gently. The gel was then slowly poured into the middle of the gel cradle with appropriate comb and allowed to solidify for 30 min at room temperature. The gel was later transferred to frame and covered with 1x TAE buffer. Afterwards, RNA samples were prepared for loading into gel by mixing 2 µl RNA sample with 3 µl TAE buffer and 1 µl of 6x loading buffer on a parafilm. To quantify the size of the bands, 3 µl of HyperLadder 1kb size ladder (Bioline) was loaded in the first well. The subsequent wells were loaded with RNA mixture (6 µl total) in each well. The voltage supply was connected and voltage set to 80 V and the gel allowed to run for 40 min. Finally, gels were examined under UV illumination using a Safe Imager™ blue-light transilluminator and/or a Chemi Genius 2 BioImaging System (Syngene). Images were visualised, analysed and photographed using GeneSnap image acquisition software (Synoptics Ltd). The same procedure was followed for assessing the quality of qPCR products formed. The sharpness and ratio of the ribosomal bands (28S rRNA to 18S rRNA) were evaluated to confirm that the extracted RNA was intact and not degraded. As the 28S rRNA should be approximately twice as intense as the 18S rRNA band, the ratio of 2:1 (28S:18S) is good indication of intact RNA (Nolan et al. 2006). DNA integrity was assessed based on the structure of the band and in comparison with the molecular size ladder.

2.7.3 qPCR Primers

Highly purified salt-free primers for reference and target genes were ordered from Integrated DNA technologies (IDT, Australia). Tm of primers ordered from IDT were in the range of 59-61^{o}C. The qPCR primers for the two reference (cytoplasmic ribosomal protein, *L2*; elongation factor-1 alpha, *EF1*) and target genes (*FER3, IRT1*) used in the present study were as used in earlier reports (Nicot et al., 2005 and Legay et al., 2012) on gene expression studies in potato during stress conditions. Primers were designed with the Primer3 (Legay et al., 2012) and the Primer Express (v2.0) Applied Biosystems software (Nicot et al. 2005) with the following criteria: primer size between 18 and 25 base pairs, GC content of 40 and 60%, amplicon size between 60 to 150 base pairs. Matching primer sets were validated using NetPrimer and the specificity of primers were tested in a silico BLAST engine (Legay et al., 2012). Nicot et al. (2005) verified the sequences of the primers through purification, cloning and sequencing of amplification products. These sequences were then compared to GenBank sequences used to design primers with BLAST 2 sequences software (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and were found to have 100% identities with GenBank sequences. The references genes are those selected to be the two best and most stable reference genes using the Normfinder tool (Nicot et al., 2005; Legay et al., 2012)

2.7.4 RT-qPCR reactions

The gene expression analysis was performed with KAPA SYBR® FAST qPCR Kits (Kapa Biosytems, Boston, USA) using Rotor-Gene Q (Qiagen). The reaction is based on the ability of SYBR green to bind to double stranded DNA and fluoresce allowing the quantification of DNA. Total reaction volume of 10 µl consisting of 5 µl of KAPA qPCR buffer (Kapa Biosytems, Boston, USA), 1 µl of each forward and reverse primer, 2 µl of sterile millipore water and 1 µl of cDNA was used. The thermal-cycle consisted of initial hold at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing of primers at 60°C for 15 s and extension at 72°C for 20 s and melt of 72°C to 95°C, raising 1 degree each step and 5s wait for each step afterwards after 90s of pre-melt conditioning on first step. The accumulation of the PCR product is measured in terms of Relative Florescence Units (RFU). Where multiple samples were assayed, a “master mix” with/ without template and primers were prepared based on the objective for qPCR analysis. A standard volume of this “master mix” was aliquoted into each reaction well to reduce technical errors and minimise the number of pipetting steps.

2.7.5 qPCR amplification efficiencies

A four-fold dilution series (10^{-1}-10^{-4}) of cDNA (a pool of all available cDNA samples to mimic as much as possible the actual samples to be measured) was used in triplets for the determination of qPCR efficiency of each primer pair/gene. Logarithms of serially diluted cDNAs versus quantification cycles (Cq) values were plotted to evaluate the PCR efficiency from the standard curves generated for reference and target genes. The qPCR efficiencies were calculated from the slope, according to the equation, $E\% = (10^{[-1/slope]}) \times 100$ (Pfaffl, 2001; Bustin, 2000). The slope was computed from a linear regression model against the Cq values of the serial dilutions of cDNA.

2.7.6 Normalisation with reference genes

Reference genes are used as endogenous controls to normalise the data by correcting for differences in quantities of cDNA used as template (Gutierrez et al., 2008, Vandesompele et al., 2002). To attain accurate normalisation, two reference genes, *L2* and *EF1*, were used for gene expression studies. The reference genes were compared for their expression stability over different cDNA samples in triplicates. To minimise the possible errors associated with the use of one reference gene for normalisation, the geometric mean of two reference genes was calculated (Dhandapani, 2014, Song et al., 2012), and its correlation to the target gene was compared to each gene of interest.

CHAPTER THREE

Development and activation of typical Strategy I reaction mechanisms in calli in response to Fe-deficiency

3.0 Introduction

Mineral stresses (deficiencies or excesses) constitute a key constraint to crop production worldwide. *In vitro* culture offers a remarkable tool for examining the morphological, biochemical and molecular adaptations and/or changes in plant in response to nutrition and environmental stresses. The functions of mineral nutrients are normally of a cellular nature and therefore are just as critical to cultured cells as to whole plants. Cellular mineral nutrition and mechanisms by which plants resist mineral stress indicate that resistance mechanisms that will function in whole plants can be selected (Meredith, 1984).

A continuous supply of bioavailable iron is required for plant growth. Plants have evolved a conserved set of coordinated responses that ensure the maximal uptake and utilisation of iron so that iron levels within cells are balanced. The responses are altered according to the iron status in the plants. To facilitate iron uptake in an iron deficient environment, most plants evolve mechanisms to solubilise and transport iron. Under conditions of low iron bioavailability, plants utilise specialised responses to enhance Fe uptake so that adequate amount of Fe can be mobilised for key cellular metabolic processes (García-Mina et al., 2013; Hindt and Guerinot, 2012; Thomine and Vert, 2013). The responses include sensing the levels of bioavailable iron, mobilisation of iron in the root epidermis coupled with the utilisation, distribution and storage of iron in specific cellular compartments for vital biological processes (Ivanov et al., 2012; Roschzttardtz et al., 2011, 2013). Plants have developed tightly regulated iron homeostasis mechanisms to prevent cells from iron-mediated oxidative damage resulting from excess iron or its deficiency within cells (Darbani et al., 2013; Kim and Guerinot, 2007). Hence, in living organisms, Fe buffering and storage are necessary mechanisms to manage iron-mediated oxidative stress. An Fe-efficient plant is able to grow in low Fe environments (De la Guardia and Alcántara, 2002) through the activation of specific Fe-stress root responses that facilitate the uptake of Fe from the soil (Walker and Connolly, 2008; García-Mina et al., 2013). Potato, a member of the dicotyledonous family of plants, employs the reduction-based strategy (Strategy I) for the acquisition of Fe. The Strategy I approach involves proton extrusion to make iron more

soluble, reduction of Fe(III) to Fe(II) through ferric chelate reductase and the transport of Fe(II) into roots by the iron-regulated transporter, IRT1 (Robinson et al., 1999; Eide et al., 1996; Korshunova et al., 1999; Jeong and Guerinot, 2009).

Morphological disparities can be due to biochemical differences resulting from variation among expressed proteins (Bairu et al., 2011). The use of different biochemical tests to discriminate between somaclones is a valuable approach to assess somaclonal variants. It was hypothesised that biochemical processes associated with iron uptake and utilisation mechanisms in the somaclonal variant potato cells might be altered during growth under iron stress conditions compared to growth under normal Fe conditions. The key traits of interest that might be exhibited by the variant cells would be those that enable them to absorb iron from a low Fe culture medium and possess enhanced tolerance to deficiency associated problems. This chapter provides details of the various assays employed to investigate the morphological and biochemical responses of potato callus cells to Fe deficiency stress and discusses the results obtained.

3.1 Methodology

This was an experimental research in which the concentration of the variable, FeNaEDTA, was manipulated (treatments) and the effects on morphological and biochemical characteristics (dependent variables) of calli were assessed. The control groups were calli that had received neutral intervention (i.e. grown in culture medium with sufficient Fe, 50 µM FeNaEDTA).

3.1.1 Callus induction and proliferation

Callus was induced by culturing young leaf explants on half-strength MS medium supplemented with 3.22 µM of NAA and 1.78 µM of BA (see sections 2.2 and 2.4). The effect of iron on callus induction and growth was investigated by culturing leaf explants and calli respectively on a wide range of iron concentrations (representing deficient, normal and excess Fe levels). Experiments on callus induction on CIM supplemented with different Fe concentrations (0, 0.005, 0.05, 0.5, 5, 50, 200, 400, 800 µM) were conducted. A dose-response experiment in which calli proliferated for 8 weeks on CIM of 50 µM Fe content were subcultured in CIM of varying Fe concentrations (0-800 µM) was performed. Growth of callus cultures was sustained by subculturing at 4-weeks' intervals.

3.1.2 Evaluation of morphological parameters

To assess the effect of Fe nutrition on growth characteristics of calli, changes in callus fresh weights were evaluated. Visual observations, fresh weight and growth area measurements were determined. At least three Petri dishes each of three biological replicates were randomly selected for the weight determination. Calli were placed on a pre-weighed container on an analytical balance to determine the fresh weight. Callus growth was estimated from change in callus fresh weight after 4 (month 1) and 12 weeks (month 3) of culture. Change in fresh weight was calculated as:

final weight of callus (after a period of culture on growth medium) – initial weight of callus at the start of culture…….... (1)

The area of growth covered by callus was calculated using the ImageJ (v1.45) software and expressed in terms of square mm.

Changes in morphology (texture, structure and colour) were recorded on the basis of visual inspection of the callus cultures. Somaclonal variants were identified based on visual inspection for modifications in chlorophyll pigmentation.

3.2 Biochemical Assays

Biochemical analyses carried out using callus samples were as detailed in Chapter two (Section 2.6).

3.3 Sampling of callus samples

Two to three calli per Petri dish were sampled per treatment for analysis. Two to three Petri dishes (composed of 6-9 biological replicates) were randomly collected for sampling per assay. Sampling was done at 4 and 12-weeks interval and/or at the time (4-5 months) Fe-efficient (chlorosis tolerant) somaclonal variants were isolated based on visual screening. For fresh weight determinations and growth area measurements, at least three Petri dishes each of three biological replicates were randomly selected and weighed. The experiments were independently repeated three times. Prior to FCR assay, DAB and PPB staining, callus samples were soaked in 1mM Na_2EDTA for 5-10 min to eliminate traces Fe present in growth medium attached to calli followed by three times washes with MiliQ water to reduce excess EDTA (Kabir et al., 2015; Ojeda et al., 2004). For enzyme assays, calli were sampled into Eppendorf tubes, flash-frozen and stored at -80^{o}C for later extraction and further analysis. For POD enzyme assays, three extracts were prepared and 2 assays carried out per

treatment. APX and CAT analyses were carried as triplicate assays per duplicate extracts on each Fe treatment applied to callus. For chlorophyll, carotenoid and total phenolic determination, three acetone extractions were prepared per treatment and two assays were run per extract.

3.4 Statistical analysis

For the various experimental datasets, each data point represents the calculated means of three independent replicates and six biological replicates. IBM SPSS (version 23) software was used for statistical analysis. The mean percent of leaf explants forming callus, mean weight of calli, chlorophyll and carotenoid concentrations, FRO, CAT, APX and POD activities and phenolic content were the variables. Normality tests were run to ascertain the distribution of each set of data. Parametric or non-parametric statistical test methods were used based on the outcome of the normality test. Mean differences of the variables among treatments and over different culture periods (1 and 3 months) were examined using one-way analysis of variance (ANOVA) and t-test respectively. The mean differences were compared by Least Significant Difference (LSD) and Duncan tests. The level of significance for all the statistical tests was set at 5%. Correlation and binary logistic regression analyses were done to determine the relationship between variables and the associations between exposure to Fe and outcomes. Principal component and cluster analysis were performed to determine variable correlation with treatments. Pearson's correlation coefficients with the separate principle component axes were calculated for the relevant variables.

3.5 Preliminary findings

3.5.1 Effect of plant growth regulators on callus induction

Callus induction and growth was optimised to ascertain and develop the most favourable culture medium for inducing callus and sustaining its growth. In theory, equal amount of cytokinin and auxin promotes callusing, but this differs in practice probably due to the variation in endogenous level of phytohormones (Kumar et al., 2014). Induction of primary callus differed among types of media used. It was observed that the percentage of explants forming callus, callus colour and degree of callus formation varied with culture medium formulations (Table 4).

After a week of culturing leaf explants, callus had started to form in NB10, NB17, NB18, NB34, NB27 and NB42 media (Table 4). Within 3 weeks, callus growth had increased gradually or considerably in these media. Compact calli formed were generally green in colour, solid and hard in texture. The highest percentages (56.25 for week one and 90.42 for week 3) of explants forming compact callus were recorded for half-strength MS medium supplemented with 3.22 µM NAA and 1.78 µM BA (NB10). Primary callus induction in NB10 medium was significantly ($p<0.05$) higher compared to all other culture medium formulations. This medium was found to be optimal for callus induction: it induced callus formation most rapidly and the degree of callus formation was best on this medium after 3 weeks and beyond compared to the other callus induction media. For the other 17 culture medium formulations (see Appendix Table A3), callus development was either largely ineffective or observed mostly after 4 weeks of culturing leaf explants. Furthermore, some CIM resulted in the development of a mixture of calli and shoots or extensive root structures. Media containing only NAA (N1-7) or BA (B1-7) and those without PGRs (MS medium) did not induce callus formation even after prolonged culture periods (8-12 weeks). Such media were neither considered nor chosen for further experiments on callus induction.

Table 4. Effect of different combinations of NAA and BA on callus induction from potato leaf explants.

culture medium	plant growth regulators		% of explants formed callus		degree of callus formation
	NAA (µM)	BA (µM)	1 week	3 weeks	
½ MS	0	0	0.00^{e}	0.00^{f}	–
N	1.07-10.74	0	0.00^{e}	0.00^{f}	–
B	0	0.89-8.88	0.00^{e}	0.00^{f}	–
NB10	3.22	1.78	56.25 (±6.25)^{a}	90.42 (±3.15)^{a}	†††
NB17	3.22	2.66	31.25 (±6.05)^{bc}	70.83 (±3.61)^{b}	†††
NB18	4.30	2.66	37.5 (±6.25)^{b}	52.08 (±3.6)^{c}	††
NB25	4.30	3.55	14.58 (±4.0)^{d}	41.67 (±3.08)^{d}	††
NB51	5.37	0.44	22.92 (±3.61)^{cd}	60.42 (±4.0)^{c}	††
NB34	8.06	4.44	31.25 (±6.0)^{bc}	33.33 (±3.60)^{d}	†
NB27	8.06	3.55	33.33 (±3.0)^{b}	37.5 (±6.25)^{d}	†
NB42	10.74	6.66	50 (±6.25)^{a}	58.33 (±7.21)^{c}	††
NB43	1.07	8.88	18.75 (±5.25)^{d}	39.58 (±9.55)^{d}	†
NB7	10.74	0.89	0^{e}	10.42 (±3.0)^{e}	†

† ≡ slight callus, †† ≡ Moderate callus, ††† ≡ Massive callus

Callus colour: light green (in week 1), green - very green (in week 3)

Data obtained from all experiments are presented as the mean ± SD of three independent replications. Means having different letters within a column are significantly different as indicated by LSD and Duncan Post-hoc multiple comparison test $P<0.05$.

3.5.2 Effect of Fe supply on callus induction and growth

Morphological characteristics of calli were monitored to ascertain whether the exposure of callus to Fe stress conditions generated differences in the physical and/or visual appearance of callus. Growth of callus under the different Fe supply conditions (0-800 µM) was monitored to investigate the effect of iron on callus growth as well as to determine the lethal and sublethal inhibitor concentrations of the selective agent (Fe). In general, subcultured calli proliferated at a reasonably faster rate than primary calli (from leaf explants) growing on CIM of 0-800 µM Fe (Figure 6). More subcultured calli thrived and increased in area of growth at 0-400 µM Fe concentrations compared to callus induced from leaf explants on the same media. Chlorotic symptoms and changes in pigmentation (yellow to light green at Fe

deprived and low Fe levels; green to very green at sufficient to high Fe amounts) were observed (Figure 14) in primary calli within 3-4 weeks but from 6-8 weeks in subcultured callus. Survival and growth of callus on 400 µM of Fe was lowered compared to calli growing on the control, 50 µM Fe medium. Calli and leaf explants appeared to be in the final stages of senescence and became necrotic on 800 µM Fe medium - colour changed from the initially green colour to brownish black (Figure 6)**.** The lethal and sublethal Fe concentrations for callus induction and growth were found to be 800 and 400 µM respectively.

It was presumed that the absence or presence of Fe in culture medium could respectively inhibit or promote callogenesis. It was discovered that the omission of Fe from CIM did not prevent callus induction from leaf explants nor growth of subcultured callus. The resultant calli were light green, yellow to cream in colour (largely chlorotic). Upon continuous deprivation of Fe supplies, callogenesis and proliferation was slower and colour of callus was usually light yellow to cream or brown. Excess levels of Fe however, resulted in inhibition of callogenesis and caused necrosis in the subcultured calli (see Figure 6). The effects (inhibition of callus induction and proliferation, senescence, necrotic patches) of lethal Fe concentration were detected more rapidly (in 1-2 weeks) in leaf explants or primary callus than in the subcultured calli in which the effects were evident from the 3^{rd} - 4^{th} week. Fe stress (excess or lacking) causes growth and size reduction, senescence, necrosis and even plant death (Chaterjee et al., 2006; Sperotto et al., 2008; Tewari et al., 2013). It could be inferred from the current study that the leaf explants possibly provide a larger surface for interaction with medium and hence, could be more sensitive in their response to Fe stress levels than the calli.

The initial size of callus subcultured was detected to influence the rate of growth: larger calli grew faster than smaller ones and seemed to be less affected under comparable culture conditions. Both sizes and weights of calli were conveniently obtained by the use of a cork borer and a weighing balance. The rate of increase in growth and the size of primary callus cultures were observed to be affected by the age (young leaf) and/or size of leaf cultured. There was some degree of disparity in the sizes of leaves of micropropagated stock plants available for use. It was almost impossible to obtain leaves of the same size, at the same stage of development (young) and in large enough quantities for all experiments. Based on the aforementioned challenges and the fact that subcultured and primary calli exhibited similar responses (in terms of alterations in pigmentation and development of chlorotic symptoms) to Fe-deficiency, further studies on the responses of callus cultures to Fe supply were carried out using subcultured calli.

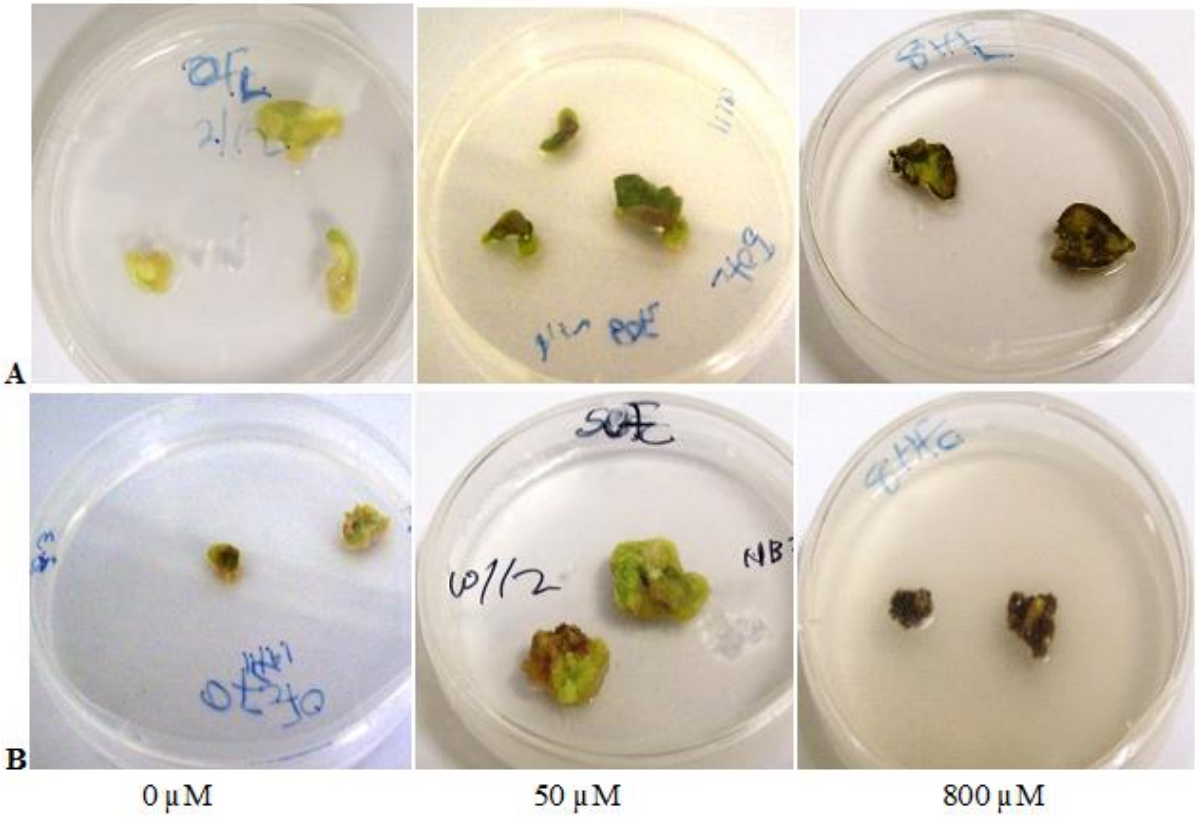


Figure 6. Leaf explants (A) and callus (B) on culture medium without Fe (0 µM), with sufficient (50 µM: control) and lethal Fe (800 µM) concentrations.

3.6 Main results and discussion

3.6.1 Morphological response of callus cultures to Fe supply

3.6.1.1 Fe-deficiency caused retardation in callus growth

Callus weight and the growth area covered by callus were used as a preliminary evaluation for the response to Fe-deficiency. It was presumed that Fe stress conditions will have an effect on the growth and/size of on callus. The results (Figure 7) show that the fresh weight of callus was dependent on the amount of Fe in the growth medium. Initially (after one month), there was a stable to gradual increment in fresh weight of calli on 0-0.5 µM Fe medium, followed by a sharp rise on 5-50 µM Fe medium and then there was steep decline on 200-800 µM Fe medium. In the first month of culture, fresh weight of calli in Fe-deprived (0 µM) medium was about three folds less than calli on control (50 µM) medium. After one month,

differences in the mean fresh weights between calli growing on 0-0.5 µM and 200 – 400 µM Fe medium were not significant ($p>0.05$).

Following prolonged exposure (three month) of calli to varying Fe supplies, there was an increase in the fresh weights of calli with increasing Fe levels followed by a gradual decline as Fe exceeded optimal (50 µM) levels. There was a steady increase in fresh weight from 0 to 0.05 µM followed by a sharp rise from 0.5-50 µM and then a reduction in weight at excess Fe levels (200-800 µM). Generally, calli showed lower fresh weights under Fe stress (0 µM or deficiency and 800 µM or excess) treatments than at 5 to 50 µM Fe concentrations (after one month) and 5 to 200 µM (after three month). Calli growing on control medium (50 µM Fe) were found to have the highest gain in fresh weight. After one and three months, fresh weight of calli on 800 µM (lethal Fe concentration) was significantly lower by 7.5 and 54-folds respectively compared to the control (calli on 50 µM). The mean difference in fresh weight between month three and one was statistically significant at the 0.05 level. The mean weight of calli after three months was about 4-6 times greater than that after one month. A 70% increment in the mean fresh weight of calli growing on sufficient Fe medium was measured after three months compared to after one month.

Overall, the mean area of callus growth increased with a rise in the amount of Fe (0-50 µM) supplied to the medium as well as with an increase in the culture period (Figure 8). A decline in growth area was recorded at excess levels of Fe (200-800 µM). A gradual increase in the mean area of callus growth was recorded as the Fe supplies in growth medium was enhanced (Figure 7). In the first month of culture, the mean change in area of growth covered by calli on Fe-free (0 µM) and normal Fe (50 µM) medium was about 33 mm^2 (Figure 7) and this difference was statistically significant ($p<0.05$). After three months, calli on the control (50 µM Fe) medium increased in area of growth by 33%. Calli growing on 5 µM Fe had a mean area of growth about twice less than calli growing on control medium after three months of culture.

The mean difference in the area of callus growth between one and three months' culture period was statistically significant ($t=-8.472$, $df=160$, $p<0.05$). The area of callus growth increased significantly ($p<0.05$) with increasing culture durations. The area of callus growth increased significantly ($p<0.05$) with increasing culture durations. Overall, there was a 29-60% increase in the mean callus growth area after three months compared to after one month but there was a slight reduction (1.43 mm^2) in the mean area of callus growth on the medium containing 800 µM Fe. At 0.5, 5 and 200 µM Fe concentrations, a 31-36% increment in the area of callus growth occurred after three months in culture compared to one month.

The mean area of callus growth on the medium with 5 µM Fe was 33% higher after three months compared to after one month. The mean areas of callus growth on 0, 0.005 and 0.05 µM after three months was twice as much as those recorded in after one month. At 0, 0.005 and 0.05 µM Fe the mean areas of callus growth after three months were similar (289-234 mm^2) to those after one month (70-80 mm^2).

There was a wide distribution in the area of callus growth irrespective of culture duration and/or Fe concentration in culture medium. A wide range of variations in the area of callus growth as shown by the skewness, maximum and minimum value bars as well as outlier values (see Figure 8). These outliers could represent data points of sections (cells) of calli exhibiting tolerance to chlorosis and as such yielded results (values) that did not follow the typical distribution of the rest of the data set. Some calli, despite being exposed to Fe deficiency conditions, covered an area of growth similar or close to calli cultured on control medium for the same duration of time in culture. A distinctively few calli subjected to no or low Fe supplies (0-5 µM) proliferate profusely and in a similar manner to those on sufficient and excess Fe levels (50-200 µM). Such calli cells were presumed to be potential somaclonal variants capable of thriving under Fe-deficient conditions.

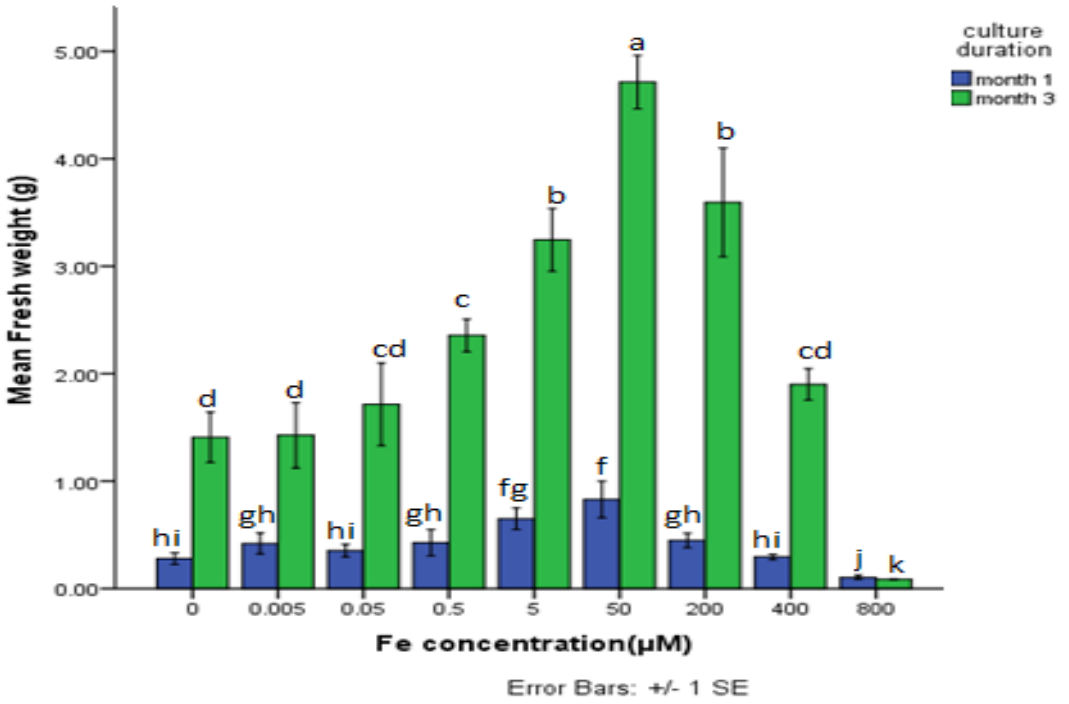


Figure 7. Fresh weight of calli cultures grown on media of varying FeNaEDTA content (0-800 µM). Calli originally proliferated on 50 µM FeNaEDTA medium were transferred to media of 0-800 µM FeNaEDTA. Means with different letters are statistically significant ($p < 0.05$).

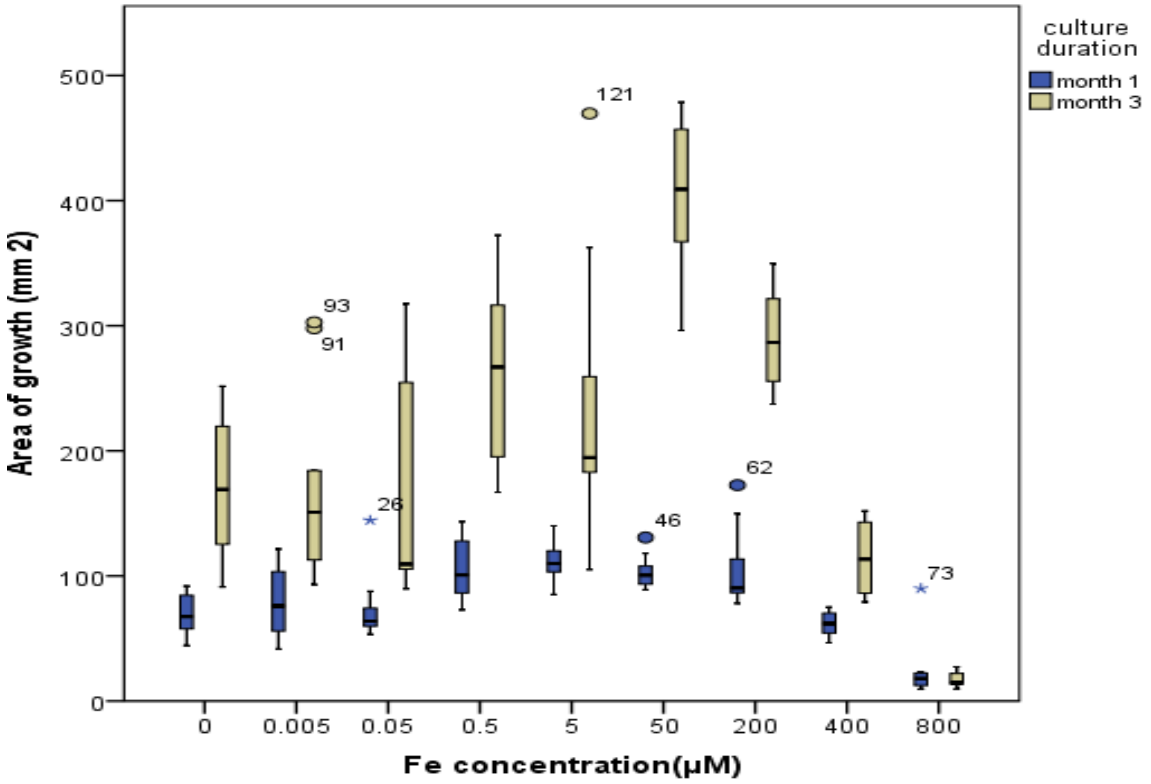


Figure 8. Effect of Fe supply on area of growth covered by callus cultures. Calli proliferated on 50 µM FeNaEDTA medium were transferred to media of varying FeNaEDTA content (0-800 µM).

Fresh weight has been shown to be a good indicator of growth. The fact that the fresh weights of calli were affected by the amount of iron supplied to the medium, suggests that iron plays a role in the nutrient stress response of the plant. In particular, the reduction in callus weight was related to iron stress (deficiency and excess) conditions. Lack of iron (0 µM) in culture medium appears to considerably affect the growth (weight) of callus compared to growth on a medium with sufficient Fe supplies (Figures 7). Piagnani et al. (2003) found that the fresh weight of callus cultured in the absence of iron was markedly lower compared to that of callus in control medium regardless of the Fe-efficiency status of the *Vitis* genotype used. Fe-deficiency also resulted in decreased dry weight in rice plants (Sperotto et al., 2008). The biomass of potato was reduced significantly under low and excess iron supply conditions as compared to control plants with a more pronounced effect at excess Fe (2.0 mM) levels (Chaterjee et al., 2006). Additionally, tuber dry weight decreased significantly under both low and excess Fe supplies. The authors discussed that the reduction in biomass might be due to disturbed carbohydrate and nitrogen metabolism and decrease in protein content. It can be inferred from the results of this study that excess Fe (800 µM) was more toxic and inhibits callus growth to a larger extent than Fe deprivation from medium (see Figures 6). Callus senescence and necrosis on 800 µM Fe-containing medium was possibly due to toxicity effects resulting from Fe-induced oxidative stress. Excess Fe is toxic since unbound iron can react with oxygen to generate free radicals that destroy cellular components including proteins, DNA, lipids and sugars (Perron and Brumaghim, 2009).

The amount of Fe in the growth medium seems to influence the area of callus growth as Fe-deficiency caused a reduction in callus growth area. The results are in agreement with Tewari et al. (2013) who reported that plants showed retarded growth with reduced cell size when deprived of iron nutrition in growth medium. Iron starvation can hinder cell proliferation because it is required by ribonucleotide reductase and other enzymes involved in cell division. Ribonucleotide reductase (RNR), an Fe-containing enzyme of universal importance, is essential for DNA synthesis (de la Guardia and Alcántara, 2002; Gruber and Kosegarten, 2002). Ribonucleotide reductase activity is dependent on the iron-supply and also regulates cell proliferation (Sanvisens et al., 2011). It is suggested that plant growth is restricted by Fe-deficiency due to the inhibition of RNR synthesis which causes nuclear and cellular division to be severely hindered (Gruber and Kosegarten, 2002; de la Guardia and Alcántara, 2002). The present study is consistent with the notion that growth depression is directly caused by insufficient Fe supplies. Also, previous reports have shown that iron is fundamental for synthesis of DNA and cell division since Fe is a constituent of RNR.

According to Gruber and Kosegarten (2002), a lack of DNA severely affected plant cell division and meristematic growth.

3.6.1.2 Fe deficiency involved with chlorosis development

To investigate visual manifestation and responses of callus cultures to Fe deficiency stress, callus growth and pigmentation were initially monitored under Fe-deficiency conditions. Visual screening and identification of chlorophyll pigmented and chlorotic regions were performed with callus cultures subjected to Fe deficiency. After one month of subculture, calli on 0 -50 µM Fe medium were actively growing (Figure 9 I). In addition, calli on 50 µM and Fe-deficient (0-5 µM) medium did not reveal a marked difference in colouration (Figure 9 I). The cells previously cultured on medium with sufficient amount of Fe (50 µM) probably had some Fe stores. Therefore, the cells might not have been severely affected (especially in terms of pigmentation) by the sudden decrease in Fe supplies upon subculture.

Major differences in the pigmentation (chlorotic symptoms) of calli cultured on Fe-deficient media were evident from the second and third subcultures (see Figure 9 II and III). Calli challenged with Fe deficiency developed the typical iron-deficiency symptom, i.e. chlorosis (cultures turned yellow) within 2-3 months in culture. The severity of chlorosis increased with the extent of depletion of Fe from the growth medium and the duration in culture. It was observed through visual inspection of calli that the colour of calli growing under Fe-deficient conditions (0 -5 µM) was yellow/light green and green in sufficient Fe (50 µM) medium after two subcultures (3 months) in the respective media (Figure 9 III). Within 3 months of subcultures, growth of most compact calli on Fe-deficient medium was slow or retarded and highly chlorotic except for putative somaclonal variants that may be tolerant to Fe-deficiency (see Figure 9 III).

The results demonstrate that the formation of the photosynthetic pigments, in calli similar to plants, is dependent on the amount of iron supplied. Insufficient amounts of Fe, has a negative effect on chlorophyll synthesis. Further proof and details of the effect of Fe deficiency on chlorosis development and biosynthesis of photosynthetic pigments (chlorophyll and carotenoid) is provided below in section 3.6.2.

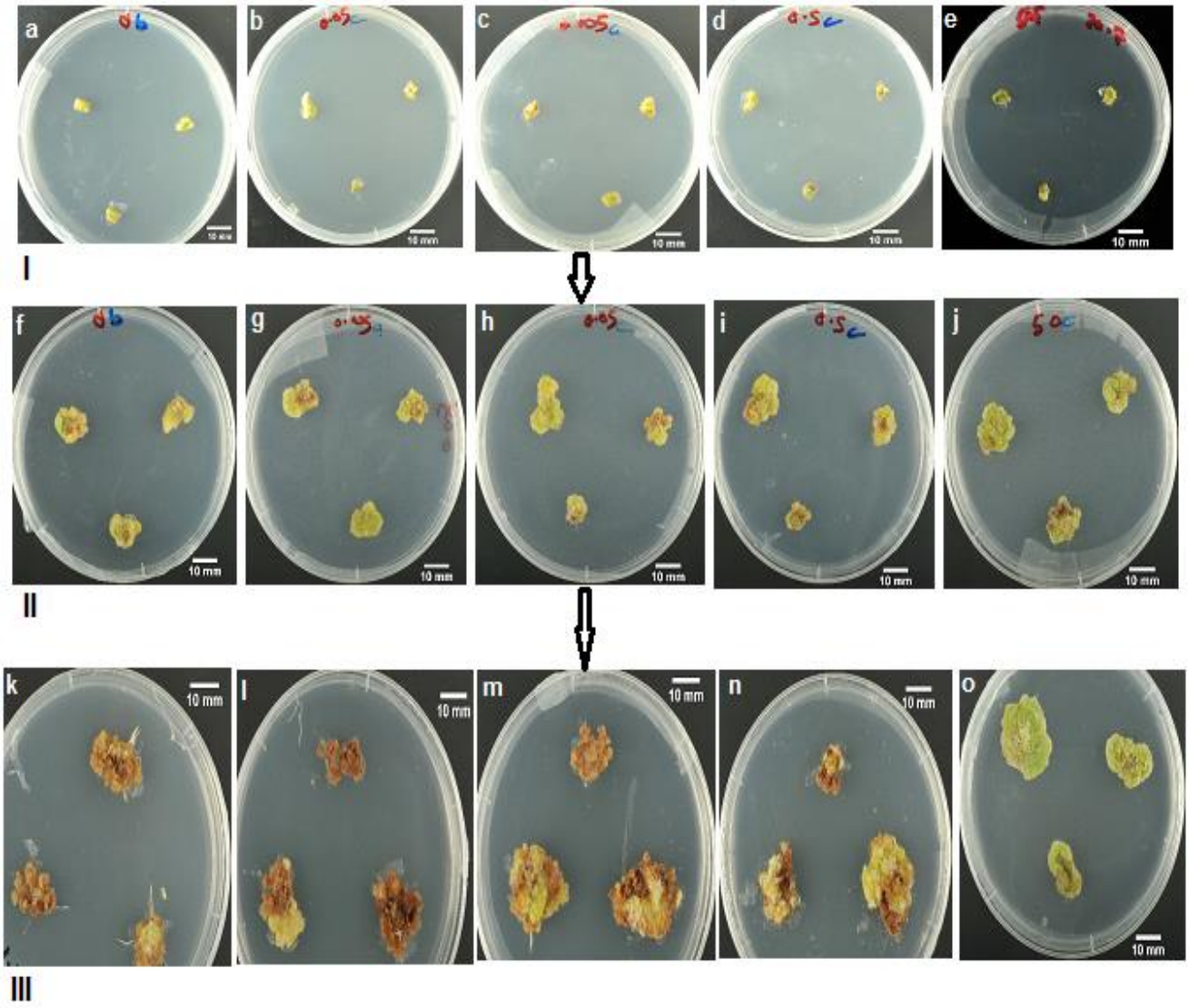


Figure 9. Changes in callus pigmentation and texture under conditions Fe deficiency (0- 0.5 µM) and sufficiency (control, 50 µM) over different culture periods. Even sizes of calli priory growing on sufficient Fe medium were transferred onto CIM of different Fe concentrations (I: day 1; a-e) over a period one month (II; f-j) and after two subcultures, three months (III; k-o).

In addition to chlorotic symptoms noticed in calli on medium with low Fe supplies, the texture (soft and brittle) and structure were also affected. Iron shortage results in the remodelling of cell wall structure and metabolism since proteins involved in the metabolism of glycosyl compounds are Fe-responsive in plant species (López-Millán et al., 2013). The possible involvement of Fe in cell wall construction may account for the modifications observed in the structure and texture of calli challenged with Fe stress (deficiency and excess)

conditions. According to Ashrafadeh et al. (2015) the nature or structure of plant callus cell wall plays an essential role in plant response to trace element stress or toxicity.

Tewari et al. (2013) detected necrotic lesions in iron-deprived chlorotic leaves at an advanced stage of iron deprivation in consonance with the current study where browning (necrosis) of calli accompanying reduced callus size were detected especially in long-term cultures. Fe-deficiency is linked to senescence pathways and can cause death in plants (Sperotto et al., 2008). Programmed cell death (PCD) is a normal physiological process to remove aged and/or damaged cells in living tissues. However, during the hypersensitive response to stress, non-autolytic PCD evidenced as necrosis may occur in plants (Tewari et al., 2013). It is suggested herein that cell death occurs in plants under conditions of severe and prolonged iron-deficiency. In the case of rapeseed leaves, Tewari et al. (2013) discussed that the immoderate levels of H_2O_2 induced by Fe deprivation in plants activates non-autolytic PCD due to a weak antioxidant defence system under such conditions. Evidence that non-autolytic PCD ensued in iron-deprived plants was backed by the results of a cell death assay which showed extensive cell death in young rapeseed leaves of Fe-deprived compared to Fe-sufficient plants.

3.6.2 Biochemical responses of calli to Fe-deficiency

Under low Fe conditions, plants can develop controlled responses to enhance Fe uptake capacity. Various biochemical assays were carried out to investigate the response of callus cultures to iron deficiency. The use of different biochemical tests to discriminate between somaclones is a valuable approach to assess somaclonal variants. Morphological disparities can be due to biochemical differences resulting from variation in gene expression (Bairu et al., 2011). It was hypothesised that biochemical processes associated with iron uptake and utilisation mechanisms in the somaclonal variant potato cells might be altered during growth under iron stress conditions compared to growth under normal Fe conditions. The key traits of interest that might be exhibited by the variant cells would be those that enable them to absorb iron from a low Fe culture medium and possess enhanced tolerance to deficiency associated problems.

3.6.2.1 Iron deprivation suppressed photosynthetic pigments

a) Chlorophyll content

Chlorophyll and carotenoid concentrations were determined to investigate whether the formation or levels of these pigments was influenced by the Fe nutritional status of calli. Also, to assess whether the levels were related to the differential pigmentation and visual chlorosis symptoms identified in calli growing on Fe-deprived, low and sufficient Fe media. There was a rise in total chlorophyll content in calli corresponded with an increase in Fe levels in culture medium (see Figure 10). Also, total chlorophyll content in calli exposed to Fe-deficiency for three months (after 2 subcultures) was lower than in calli cultured for a month under the same conditions.

Within a month of culture, mean total chlorophyll content in calli grown on sufficient levels of Fe (50 µM) was about three times that of calli deprived of Fe (0 µM) and 1.5 to 2.6-folds greater than in calli on 0.005-5 µM Fe-containing medium ($p<0.05$). Calli growing on 0.05 and 0.5 µM Fe-containing medium had equivalent mean chlorophyll contents. The initial gradual rise in chlorophyll content was correlated with an increase in Fe levels in growth medium. Three-month old calli displayed a similar trend of rise in chlorophyll content with an increased in the amount of Fe supplied to the medium. The mean total chlorophyll content in calli cultured on 50 µM Fe was considerably increased (by a factor of 10, $p<0.05$) relative to that of calli on 0 µM Fe medium. Total chlorophyll content in calli on 50 µM Fe medium was 3-5 times greater than calli on 0.005-5 µM Fe-containing medium.

Overall, the average total chlorophyll content in calli after one month calli was 5% more than that for calli that had been exposed to the same conditions for three months. After one month, calli on 0-5 µM Fe medium had mean total chlorophyll content of 1 to 2-folds higher than that of month 3 calli. Contrarily, under sufficient Fe conditions, total chlorophyll content was enhanced over prolonged period in culture. The mean chlorophyll content in calli on Fe-sufficient medium after month one was 20% less ($p<0.05$) than after three months.

Chlorophyll content declined when calli were exposed to no or low amounts of Fe over a prolonged culture period but in some instances, the possible occurrence of somaclonal variation in callus cells resulted in chlorophyll content recorded after three months being equivalent to that of after one month and thus their chlorophyll content did not decrease in response to Fe-deficiency conditions. Also, potential Fe-efficient somaclonal variants tolerant to Fe chlorosis were greener and had higher total chlorophyll content than other callus cells

on the same Fe-deficient medium (see Figure 10 e.g. outlier value for month 1 calli on 0.005 µM Fe medium).

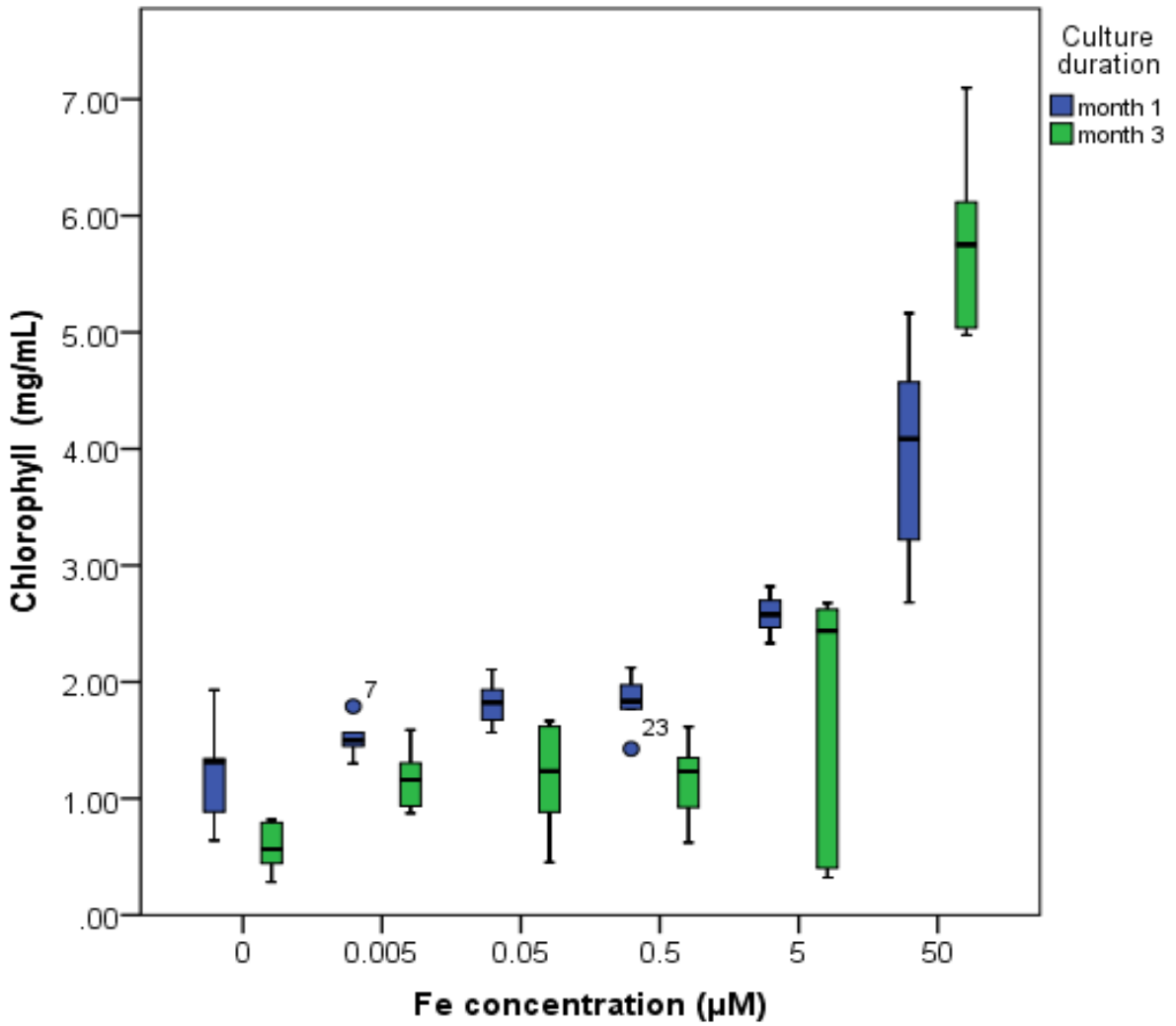


Figure 10. Effect of short and long-term exposure to Fe deficiency on chlorophyll contents of potato callus cultures. Calli initially proliferated on 50 µM FeNaEDTA medium were transferred to Fe-deficient (0-5 µM) and sufficient (50 µM) media for one month (month 1) and subsequent subcultures at 4-weeks intervals for up to three months (month 3).

b) Carotenoid content

Carotenoid content in calli cultured on medium deprived of Fe was reduced compared to the control with sufficient Fe (Figure 11). In the first month of culture, carotenoid content in calli increased gradually as Fe content in medium increased up to 0.05 µM but there was a reduction in calli cultured on media supplemented with 0.5 and 50 µM Fe (Figure 11). At a

later growth stage (three months), carotenoid content in calli under Fe-deficient conditions was significantly reduced ($p<0.05$). The mean difference in carotenoid content among 60% of the calli exposed to Fe-deficiency for three months were similar but lower ($p<0.05$) compared to the control. The longer the culture duration, the lower the carotenoid content in calli exposed to Fe-deficiency conditions. The mean carotenoid content in calli grown on Fe-deficient medium was generally significantly lower ($p<0.05$) after three months than after one month. Calli on 50 µM Fe medium was found to have a 7.5% increase carotenoid content after three months of culture compared to after one. In all, there was a 14% difference in the mean carotenoid content in calli between months one and three ($t=3.617$, $df= 70$, $p= 0.001$).

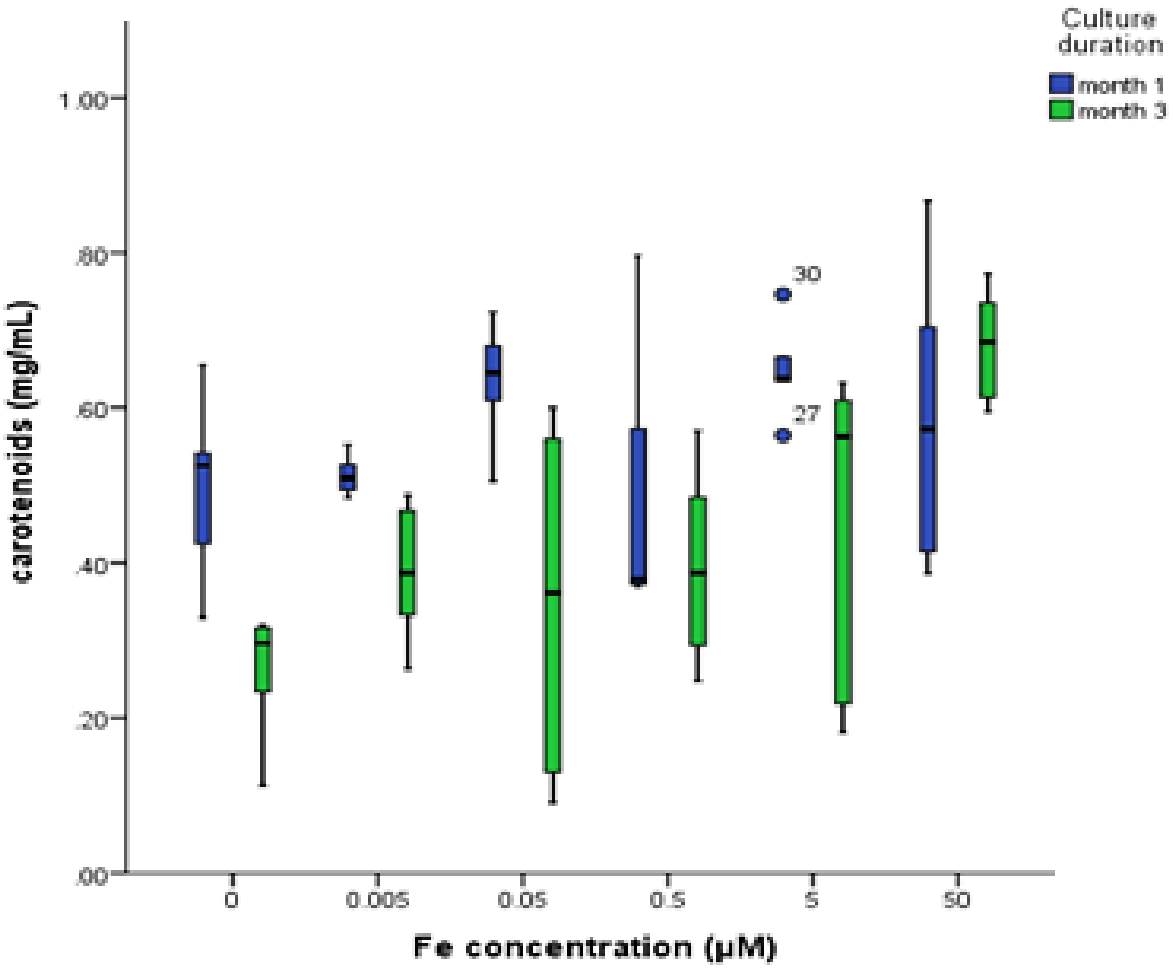


Figure 11. Effect of short and long-term exposure to Fe deficiency on carotenoid content of callus cultures. Calli initially proliferated on 50 µM FeNaEDTA medium were transferred to Fe-deficient (0-5 µM) and sufficient (50 µM) media for one month (month 1) and subsequent subcultures at 4-weeks intervals for up to three months (month 3).

Iron is a component of enzyme complexes in chloroplasts and mitochondria and is known to be required for biosynthesis of the pigments, chlorophyll and carotenoid (Tewari et al., 2013; Salama et al., 2009; Simko et al., 2008). It is, therefore, of interest to investigate if the quantities of these pigments in calli are affected by the amount of iron supplied in growth medium. Chlorophyll and carotenoid contents were detected to be dependent on the bioavailability of Fe. The results show that generally, the total chlorophyll content in calli increased with an increase in iron supply. It is suggested that chlorophyll content and/ or synthesis in calli is favoured by high Fe supplies to growth medium.

Exposure to Fe-deficiency has a negative effect on total chlorophyll content in calli and this impact is magnified as the culture period progresses. The higher chlorophyll content in calli under Fe-deficiency conditions for one month compared to that after three months could be attributed to the possible acquisition of appreciable Fe stores during prior growth of calli on sufficient Fe medium. Hence, calli were capable of sustaining chlorophyll biosynthesis during short-term exposure to low Fe supplies. After 2 subcultures (three month) however, the calli cells were probably severely to moderately depleted of Fe and as such, chlorophyll synthesis was inhibited. Tarrahi and Rezanejad (2013) reported a build-up of chlorophyll in callus cultures of rose plants with increment in the duration of culture. Fe deficiency led to decreased chlorophyll content in *C. oblonga* with the effect being more pronounced over prolonged culture period (Dolcet-Sanjua et al., 1992). In the present study, the longer calli were challenged with Fe-deficiency, the lower the chlorophyll content measured but chlorophyll content was increased under sufficient Fe conditions. This implies that continuously inadequate Fe supplies can hinder chlorophyll biosynthesis whereas ample amounts of Fe promote chlorophyll synthesis. Reportedly, iron deficiency impairs the function of the chloroplasts and mitochondria electron transport chains (Graziano and Lamattina, 2005; Salama et al., 2009). The reduction in chlorophyll content can be attributed to the function of Fe in the synthesis of the precursors (aminolevulinic acid and photochlorophylid) of chlorophyll biosynthesis (Broadley et al., 2012). Salama et al. (2009) suggested that the decrease in fresh weight accumulation may be due to the reduction in chlorophyll content and rate of photosynthesis. The aforementioned finding is concordant with the results of the study here in as illustrated in sections 3.5.1 and 3.6.1 where callus growth and weight was found to be reduced under Fe-deficiency compared to sufficient Fe conditions. According to Chaterjee et al. (2006) impairment of photosynthesis caused by decrease in chlorophyll concentration may be the cause of biomass reduction in potato.

Prolonged low Fe availability can reduce carotenoid contents in calli (see Figure 11). Tarrahi and Rezanejad (2013) reported a decrease in carotenoid content was found in calli cultured on Fe-deprived medium. Xu et al 2010 identified eight carotenoids synthesised in citrus callus and found that carotenoid composition of callus varied with genotypes. Also, the carotenoid contents were altered between subcultures. Accordingly, in this study, differences were observed between carotenoid contents in calli after one and three months of culture.

The quantities of chlorophyll and carotenoid in calli were influenced by varying Fe treatments: Fe-deficiency caused a decline in these pigments but the reverse was observed when the amount of Fe was high in the medium. There was a strong positive correlation between total chlorophyll and carotenoid content ($r = 0.753$, $p<0.001$). Reportedly, iron deficiency impaired photosynthesis: in *Brassica napus*, iron deprivation led to reduced amounts of chlorophyll and carotenoid with plants showing a decrease in growth and cell size (Tewari et al., 2013). Additionally, the carotenoid/chlorophyll ratio was higher particularly in iron-deprived plants. Accordingly, in this study, lack of Fe led to a decrease in chlorophyll and carotenoid content compared to Fe sufficiency.

3.6.2.2 Iron deficiency enhanced Fe-efficiency – FCR response

A major response of Strategy 1 plants (of which potato is a member) to Fe deficiency is the induction of FCR activity for the reduction and uptake of Fe in a readily soluble and usable form. FCR activity in calli cultured under Fe deficiency conditions was evaluated to ascertain whether FCR was stimulated in response to Fe deficiency as proposed by other researchers (e.g. Waters et al., 2002; Robinson et al., 1999). The results show that FCR activity was mostly elevated in calli subcultured on Fe-deficient media compared to those on the control (50 µM) medium (Figure 12). Generally, FCR activity was reduced as Fe supplies to the growth medium increased. Calli growing on Fe sufficient medium exhibited the least FCR activity. After one month, the mean FCR activity of calli on medium supplemented with 50 µM Fe was about twice lower than calli on Fe-deprived medium. Statistically significant ($p<0.05$) differences in mean FCR activities were recorded between calli grown on control medium and those on 0, 0.005 and 0.5 µM Fe for one month.

Calli cultured under low Fe conditions for three months showed increased FCR activities compared to calli with 50 µM Fe supply (Figure 12). The difference in the mean FCR activity between calli growing on 0 µM and 50 µM for three months was statistically significant ($P<0.05$). After three months, the mean FCR activities for calli growing on

different levels of Fe deficiency (0-5 µM) were significantly higher by 8-11% than FCR activity of calli on control medium. Slight variations existed in the mean FCR activities in calli cultured on 0-5 µM for three months but the differences were not statistically significant ($p>0.05$). A wide range of variations in FCR activity was recorded in calli after one and three subcultures (Figure 12). The overall mean difference in the enzyme activity of calli after one and three months was statistically significant ($p<0.05$).

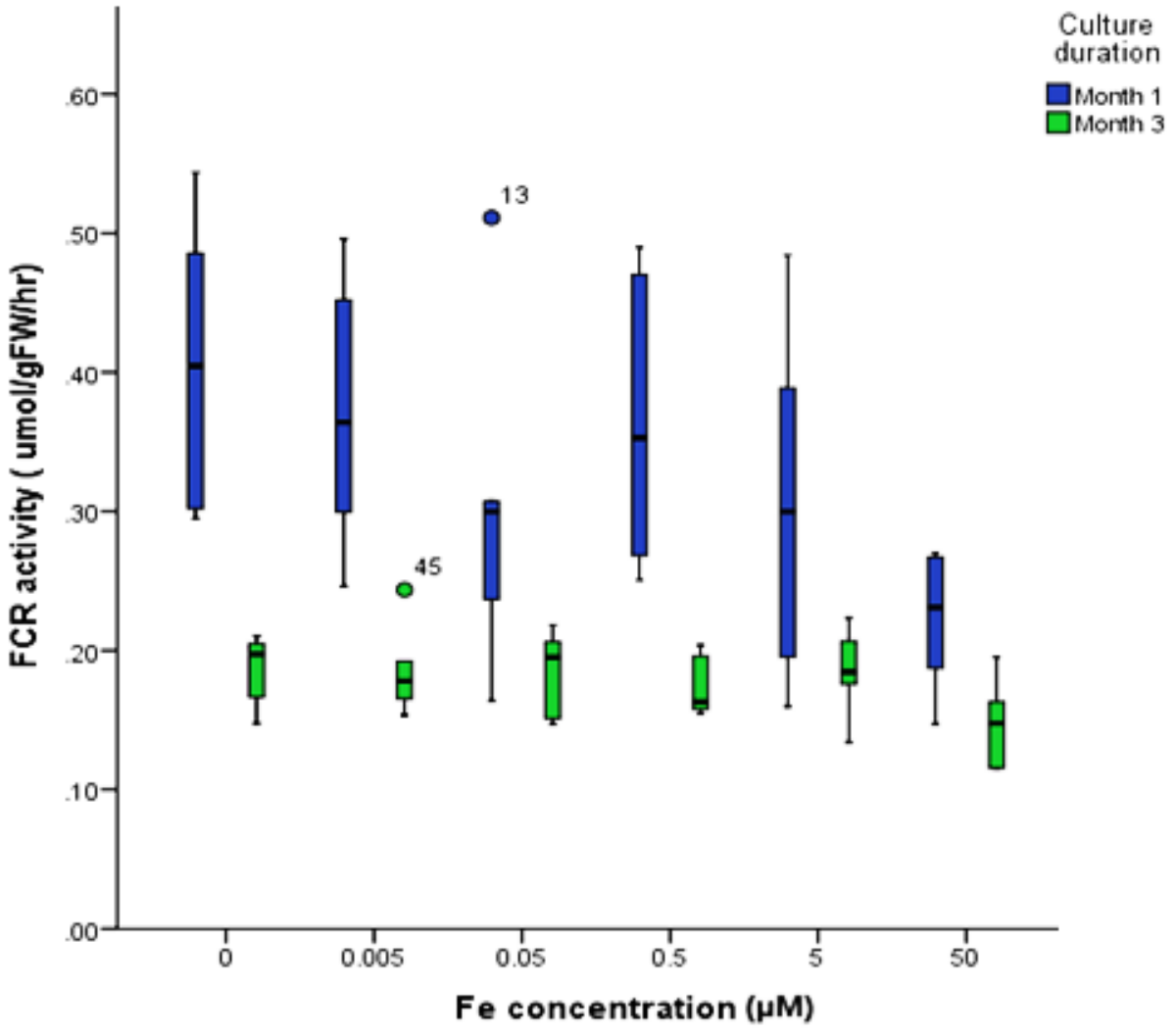


Figure 12. Effect of Fe-deficiency on FCR activity in potato calli. Calli transferred from a normal (50 µM) Fe medium to Fe-deficient media were assayed for FCR activity after the first (month 1) and subsequent two subcultures (month 3).

FCR, a member of the ferric reductase oxidase (FRO) family of metalloreductases, is responsible for root iron uptake in response to iron limitation (Robinson et al., 1999; Yi and Guerinot, 1996; Waters et al., 2002). It is, therefore, of interest to investigate the relationship between FCR activity in potato calli and iron deficiency responses. FCR activity in potato calli increased markedly in response to Fe deficiency chiefly in the first month of exposure. Correspondingly, in potato (Bienfait et al., 1987), Arabidopsis (Connolly et al., 2003) and pea (Jelali et al., 2010), FCR activities of roots were increased under all conditions of iron deficiency compared to the controls. Furthermore, Legay et al. (2012) observed a strong induction of *FRO* expression in potato under iron deficiency condition compared to controls in sufficient Fe medium. Senescence occurred in Fe-deficient calli on media mainly within three months of subcultures especially in chlorosis sensitive cells. Calli showing signs of senescence exhibited a lower FCR activity than those that retained their greenness (chlorosis tolerant) on the same Fe-deficient medium. This was most evidently depicted in the boxplot (Figure 12) by the outlier values recorded for calli growing on 0.005 µM Fe medium for three months and on 0.05 µM Fe medium for a month. The results presented herein are in consonance with the findings of Piagnani et al. (2003) which showed that FCR activity was considerably higher in grapevine calli cultured on Fe-deprived medium compared to controls, suggesting a link between FCR activity and the Fe-efficiency status of the genotype.

Somaclonal variation appeared to have increased and/or taken a pronounced effect after the first subculture of calli in media of varying Fe content. Since callus cells are closely linked, have the potential to cross feed and can form chimeras, a mixture of Fe-efficient and non-efficient cells may have been growing together and this might account for the wide distribution (high and low) of FCR activities measured in callus cultures supplied with the same amount of Fe (Figure 12). Some of the callus cells that could not adapt and/or resist the Fe deficiency stress imposed experienced retardation in growth and in some instances, were necrotic at an advanced stage of Fe-deprivation. Contrarily, some callus cells subcultured on low Fe media were actively growing similar to those sufficient Fe levels within the same culture period (month 3). Chlorosis tolerant calli showed elevated FCR activities in the third month of subculture. According to Connolly et al. (2003), over-expression of FCR (*FRO2)* makes plants capable of surviving (tolerant) on low iron growth conditions. An increased root ferric reductase activity and tolerance to iron deficiency-induced chlorosis were observed when Arabidopsis *FRO2* was over-expressed in soybean (Vasconcelos et al., 2006). It is suggested that somaclonal variations in callus cultures over time may have accounted for the

low and high FCR activities found in calli under Fe deficiency conditions compared to those in normal Fe conditions.

Potato callus cultures showed essentially the same Fe-efficiency response (high FCR) as reported in roots (Bienfait et al., 1987; Legay et al., 2012) exposed to Fe-limiting conditions. Contrary to an earlier study which suggested an absolute requirement for roots in the regulation of iron-deficient responses (Bienfait et al., 1987), this study showed that even though the potato calli did not produce roots, they displayed high FCR activity which is a key response to Fe-deficiency in Strategy I plants.

3.6.2.3 Iron deficiency induced phenolics production

The purpose of measuring the phenolic levels in calli was to find out if differences existed between those exposed to Fe-deficiency and others growing on medium with sufficient Fe. It was expected that exposing calli to low Fe media would induce phenolic production and cause an increase in the quantities measured since phenolics have been linked to enhanced Fe uptake in low Fe medium.

Total phenolic contents were determined in calli grown on both Fe-sufficient and Fe-deficient conditions. The total phenolic content in potato calli was elevated under Fe deficiency conditions (Figure 13). The results revealed that the total phenolic content in calli grown on Fe-deficient media after one month was about 1.4 to 2-folds higher than calli on 50 µM Fe-containing medium ($p<0.05$). The mean total phenolic content in calli increased slightly when cultured on media with Fe contents varying from 0 to 0.005 µM and remained virtually constant from 0.05-5 µM. In response to culture medium containing Fe concentrations of 10 and 100-folds greater than 0.05 µM had a similar effect in relation to the production of phenolic compounds.

Generally, the mean total phenolic content decreased with an increase in the culture duration (see Figure 13). The phenolic contents in calli after one and three months of culture on sufficient (50 µM) Fe medium were not significantly different **(**$p>0.05$) although a 5% decrease was observed. The mean total phenolic content in calli on Fe-deficient media was decreased by 1.5 to two times ($p<0.05$) after 2 subcultures relative to the first month. Mean total phenolics in calli after culture for one month on medium containing 0 µM Fe was about 2.7 mg GAE/g which was significantly ($p<0.05$) more than after three months.

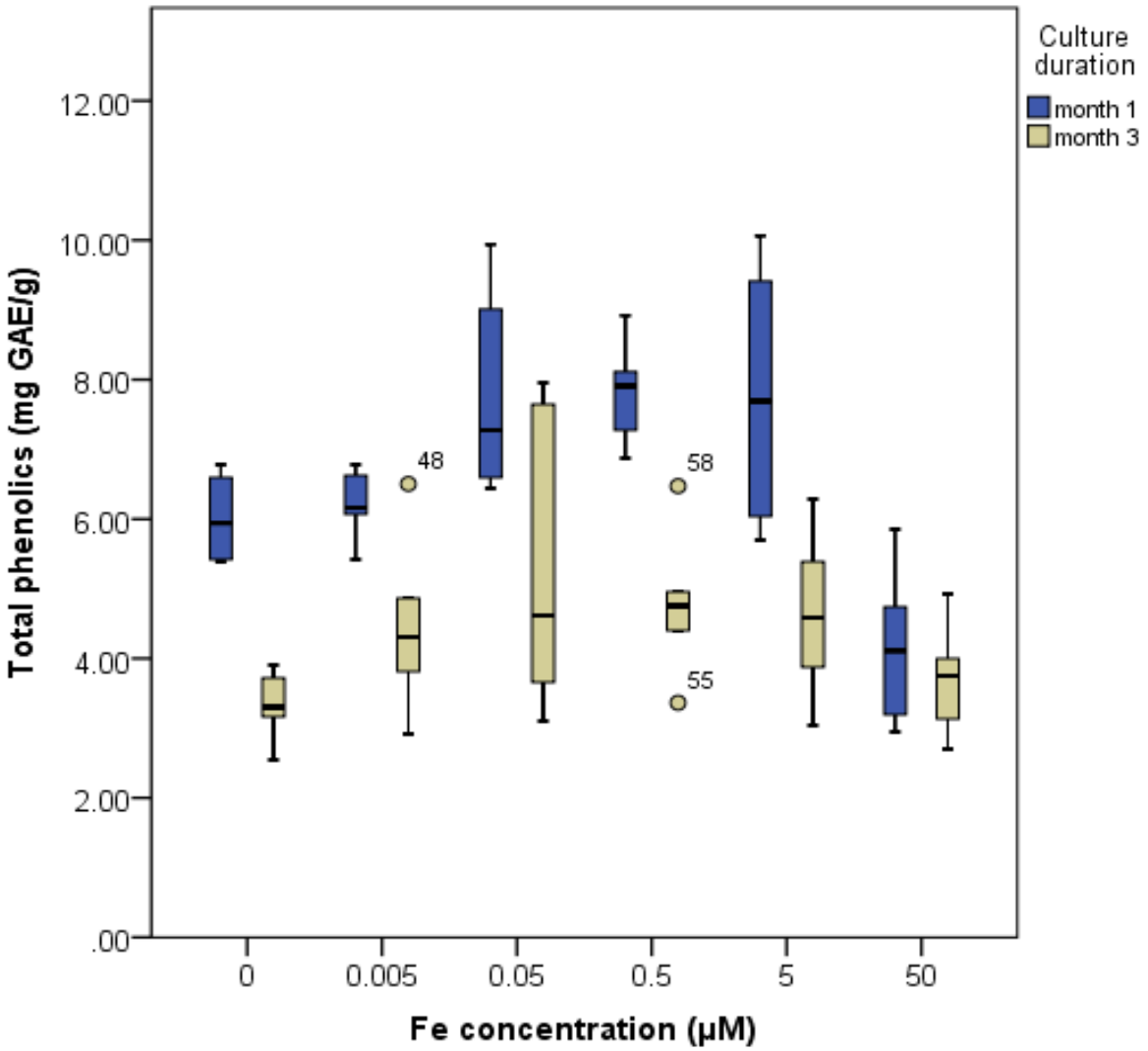


Figure 13. Phenolic content in callus cultures at an early and advanced stage of Fe deficiency. Calli firstly proliferated on 50 µM FeNaEDTA medium were transferred to Fe-deficient (0-5 µM) and sufficient (50 µM) media for 4-week duration (month 1) and further subcultures up to 12 weeks (month 3).

Phenolics are useful for Fe acquisition under conditions where Fe availability is compromised or restricted by pH or other factors (Rodríguez-Celma et al., 2011, 2013). There was an increase in the concentration of phenolics in response to heavy metal stress conditions (Wang et al., 2011; Rodríguez-Celma et al., 2011, 2013). In the present study, it was observed that although phenolics content were increased under Fe-deficiency conditions and remarkably so when deficiency was advanced (Figure 13), it appears the production of phenolics required some minimal amount of Fe. For example, total phenolics content was elevated in calli on 0.005-5 µM Fe medium compared to calli on 0 µM (Figure 13). According to Rodríguez-Celma et al. (2013) phenolics are particularly valuable when there is at least some minimal

amount of Fe such as occurs in the majority of aerobic soils or under conditions restricted by pH.

Total phenolic content in calli was elevated in response to Fe deficiency but phenolic amounts were lowered when Fe levels were increased. Similarly, increased accumulation of phenolics in potato tubers was noted to be a consequence of Fe deficiency (Chaterjee et al., 2006). Fe deficiency induces exudation of phenolics which serve as Fe-binding molecules and function in the uptake of Fe from low bioavailable Fe pools (Rodríguez-Celma et al., 2011; Jin et al., 2007). Aside from their chelating property, phenolics are considered to be involved in the reduction of Fe^{3+} (Rodríguez-Celma et al., 2011). Growth and other typical phenotypic characteristics of mutants defective in phenolics synthesis were slightly restored when mutant seedlings were grown on low Fe medium (Rodríguez-Celma et al., 2011, 2013).

The constant exposure of callus cells to Fe deficiency conditions for three months may have accounted for the significantly low phenolic content recorded. Most callus cells were necrotic after a prolonged exposure to Fe-deficiency and that could have had a negative effect on phenolic content. The generally higher phenolic content in calli subjected to Fe deficiency conditions suggests that such calli may be more efficient in utilising phenolic compounds to adapt to the stress induced and in the development of Fe-efficiency response. Fe-efficient somaclonal variants had high phenolic content relative to their counterpart chlorosis sensitive (or necrotic cells) growing on the same Fe-deficient medium. Piagnani et al. (2003) observed that high levels of phenolic compounds were produced in grape vine calli under conditions of Fe starvation, regardless of genotypes being either Fe-efficient or Fe-sensitive. Under conditions of Fe deficiency, it is presumed that secretion of iron-binding exudates, phenolics, play an essential role in the development of Fe efficiency response in plant species (Rodríguez-Celma et al., 2013; Kabir et al., 2015).

3.6.2.4 Fe supply influenced oxidative damage

The degree of Fe-induced lipid peroxidation (oxidative damage) was evaluated by measuring malondialdehyde formation (TBAR levels). Compared to Fe sufficiency, Fe deficiency had marked increased effect on lipid peroxidation in calli cultured for one month. However, the reverse was observed when Fe deprivation continued for three months (Figure 14).

After one month, there was a significant reduction ($p<0.05$) in lipid peroxidation in calli as the Fe concentration in the culture medium was increased (Figure 14). TBARS levels were higher in calli cultured on 0-0.05 µM Fe-containing media than those cultured on 0.5-50

µM Fe-containing media for one month. Generally, calli cultured under Fe-shortage conditions exhibited significantly increased mean TBARS levels relative to the control. Lipid peroxidation in about 66% of Fe-deficient calli was increased either slightly or significantly as the culture period increased. Gradually increasing TBARs levels with a rise in Fe levels was found in calli after three months of culture that had undergone two subcultures.

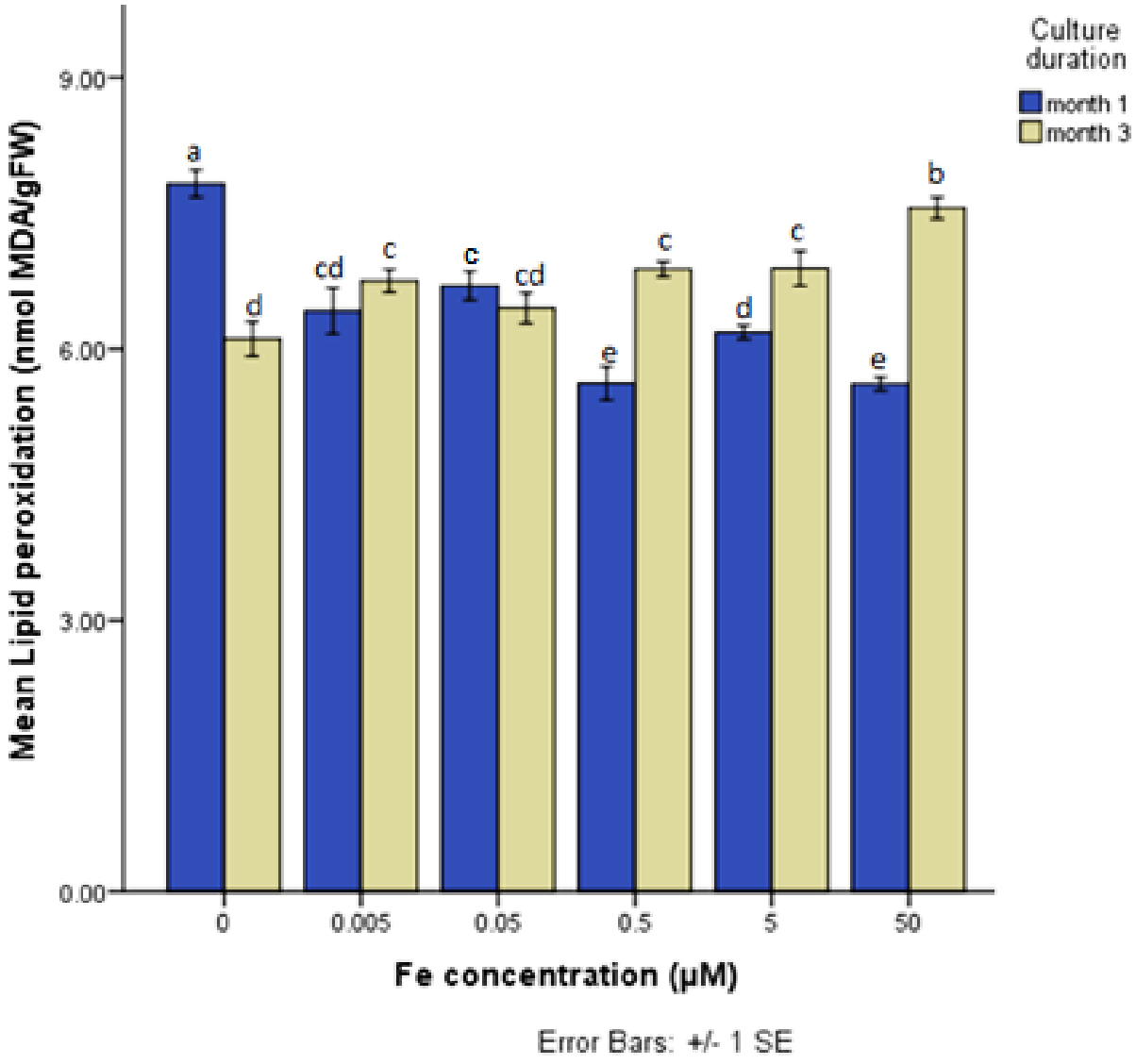


Figure 14. Lipid peroxidation (TBARs levels) in potato calli cultured on Fe-deficient (0- 5 µM Fe) and Fe-sufficient (50 µM) media for a culture duration of one (month 1) and 3 (month 3) months. Vertical bars: mean± SE. Bars carrying different letters are significantly different at 0. 05 level.

Lipid peroxidation is the oxidative degradation of lipids resulting from the overproduction of ROS and/or faulty antioxidant mechanisms. The process involves the uptake/extraction of electrons (by free radicals) from the lipids in cell membranes causing oxidative damage to cells. Lipid peroxidation of membranes is an indication of stress-induced damage at the cellular level. Considering the results for calli after three months of culture, Fe deficiency decreased TBARS levels similar to the findings of Tewari et al. (2013). It was shown that TBARS levels were correlated with the levels of iron supply and were highest in Fe-sufficient plants. Earlier findings on reduced TBARS levels in Fe-deficient plants have also been attributed to functional iron deficit (Iturbe-Ormaetxe et al., 1995; Tewari et al., 2005). Lipid peroxidation was lower in Fe-deficient calli after three months of culture and was possibly due to low availability of catalytic iron, which has been previously found to efficiently catalyse the hydroxyl radical producing Fenton reaction (Broadley et al., 2012; Halliwell, 2006; Tewari et al., 2013). Tewari et al. (2013) proposed that the oxidative stress in iron-deprived rapeseed leaves was caused more by the ROS, superoxide ($O_2^{\bullet}$), than hydroxyl ($OH^{\bullet}$) radical formation.

Contrary to the findings discussed above, Sun et al. (2007) has reported an increase in lipid peroxidation in Fe-deficient maize plants. Likewise, Fe deficiency induced considerable increase of lipid peroxidation in *Medicago ciliaris* leaves (M'sehli et al., 2014) and rice roots (Sperotto et al., 2008). A rise in lipid peroxidation activity together with membrane deterioration were identified in Fe-deficient plants (Sperotto et al., 2008). These findings signify that Fe deficiency can cause oxidative stress in plants. After one month of culture, Fe-deficient calli may have exhibited elevated TBARs levels probably due to the presence of some Fe stores (acquired during prior exposure to sufficient Fe levels) and may have had functional Fe to catalyse the scavenging or breakdown of hydroxyl radical ($OH^{\bullet}$). M'sehli et al. (2014) indicated that membrane damage can be a consequence of increased levels of H_2O_2 production, which could lead to hydroxyl radical formation and to lipid peroxidation. Basal levels of lipid peroxidation activity can occur under natural conditions as a result of metabolic processes (Sperotto et al. 2008). It is assumed that such natural occurrence of lipid peroxidation and the stress associated with growth under *in vitro* culture system may have accounted for the high lipid peroxidation observed in calli grown on Fe-sufficient medium after three months.

3.6.2.5 Involvement of Fe shortage in oxidative stress response

Reportedly, iron deficiency can lead to the production of ROS (Graziano and Lamattina, 2005; Tewari et al., 2013). To investigate this phenomena, *in situ* histochemical detection of H_2O_2 was carried out by staining callus samples with DAB. DAB is oxidised by H_2O_2 to produce a dark brown precipitate. This precipitate is exploited as a stain to identify the occurrence and distribution of H_2O_2 in plant cells. It was thought that a differential staining pattern would be detected between calli subjected to Fe-deficiency and sufficiency conditions and that the intensity of the stains may be affected by the culture duration.

The production of H_2O_2 was detected after infiltration of hand sectioned calli with DAB, which reacted with H_2O_2 in the presence of endogenous peroxidases to produce a brown polymerisation product. A differential H_2O_2 (brown) staining pattern was found between calli subjected to Fe-deficiency and sufficiency conditions (Figures 15 and 16). Callus samples without the DAB stain (negative control) were white in colour. In all, Fe-deficient calli showed a distinctly intense pattern of brown colouration. Calli on Fe-deficient media (especially at 0- 0.5 µM) generally showed the highest intensity of brown precipitates indicating that H_2O_2 production was increased under Fe-deficient compared to sufficient Fe conditions irrespective of duration in culture.

Over a three months' duration of subcultures on Fe-deficient media compared to the culture for the first month, some modifications in terms of increased stain intensity was detected mostly at 0- 0.5 µM (see Figure 16). This implies that the degree of the intensity of the stain can be affected by the culture duration. Notwithstanding, certain regions of Fe-deficient calli (0-5 µM) displayed little or no DAB stains, an indication that such sites may not have sensed stress or could represent callus cells that were potentially tolerant to Fe-deficiency and as such, not generating much H_2O_2. As the Fe content in the medium increased (from 5-50 µM), the amount of the brown stains formed was observed to be reduced (Figures 15 and 16). It can be inferred that the levels of H_2O_2 decreased with an increase in Fe supplies since calli on Fe- sufficient medium showed very minimal (in month 1) to low (month 3) H_2O_2 brown staining.

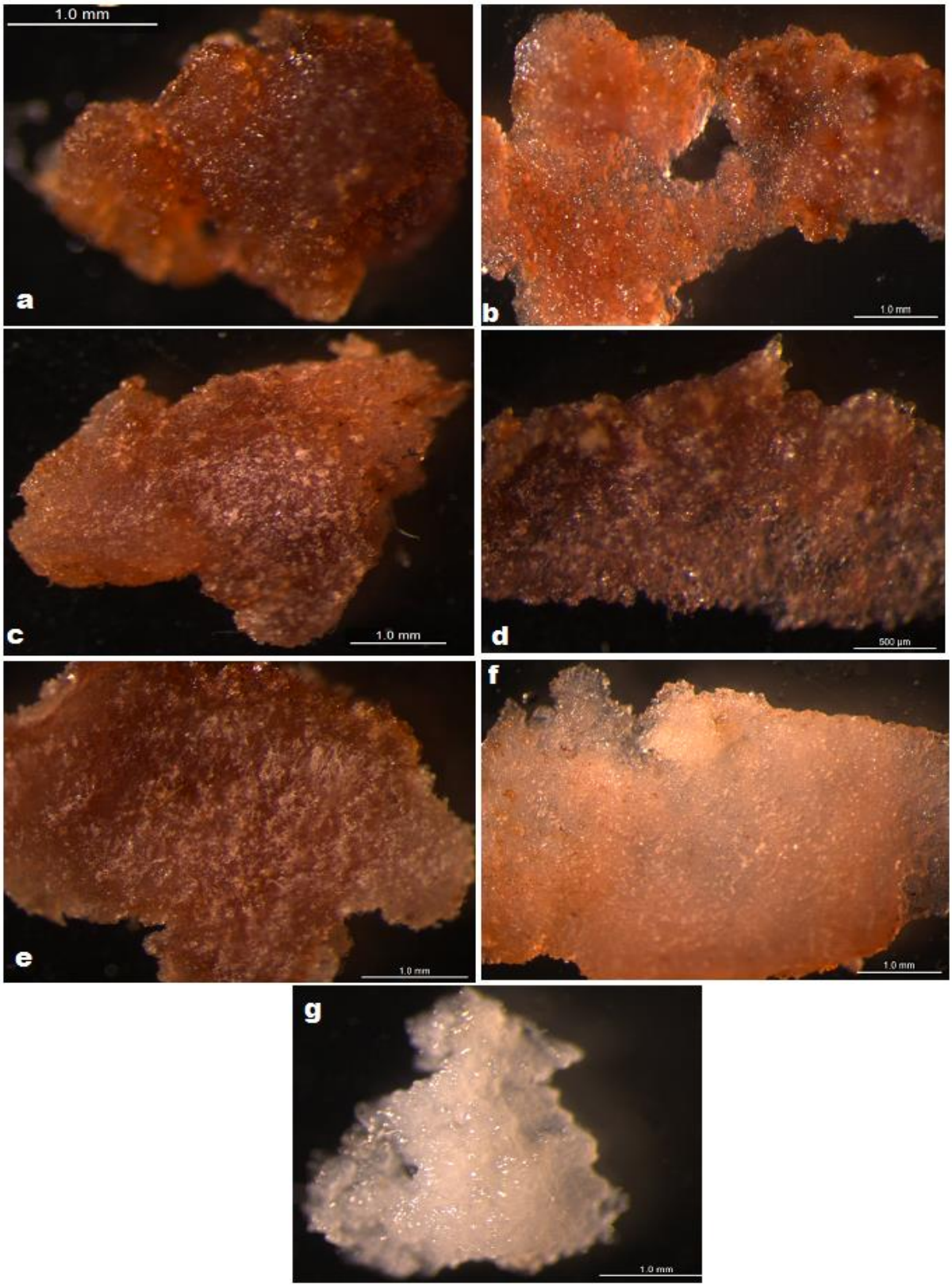


Figure 15. Histochemical staining of H_2O_2 in calli subcultured on 0 (a), 0.005 (b) ,0.05 (c), 0.5 (d), 5 (e) and 50 µM Fe media (f) for one month. Calli were subcultured on to the respective Fe-deficient media after prior growth on medium with optimal (50 µM) Fe. Callus without DAB staining is represented by letter g (negative control).

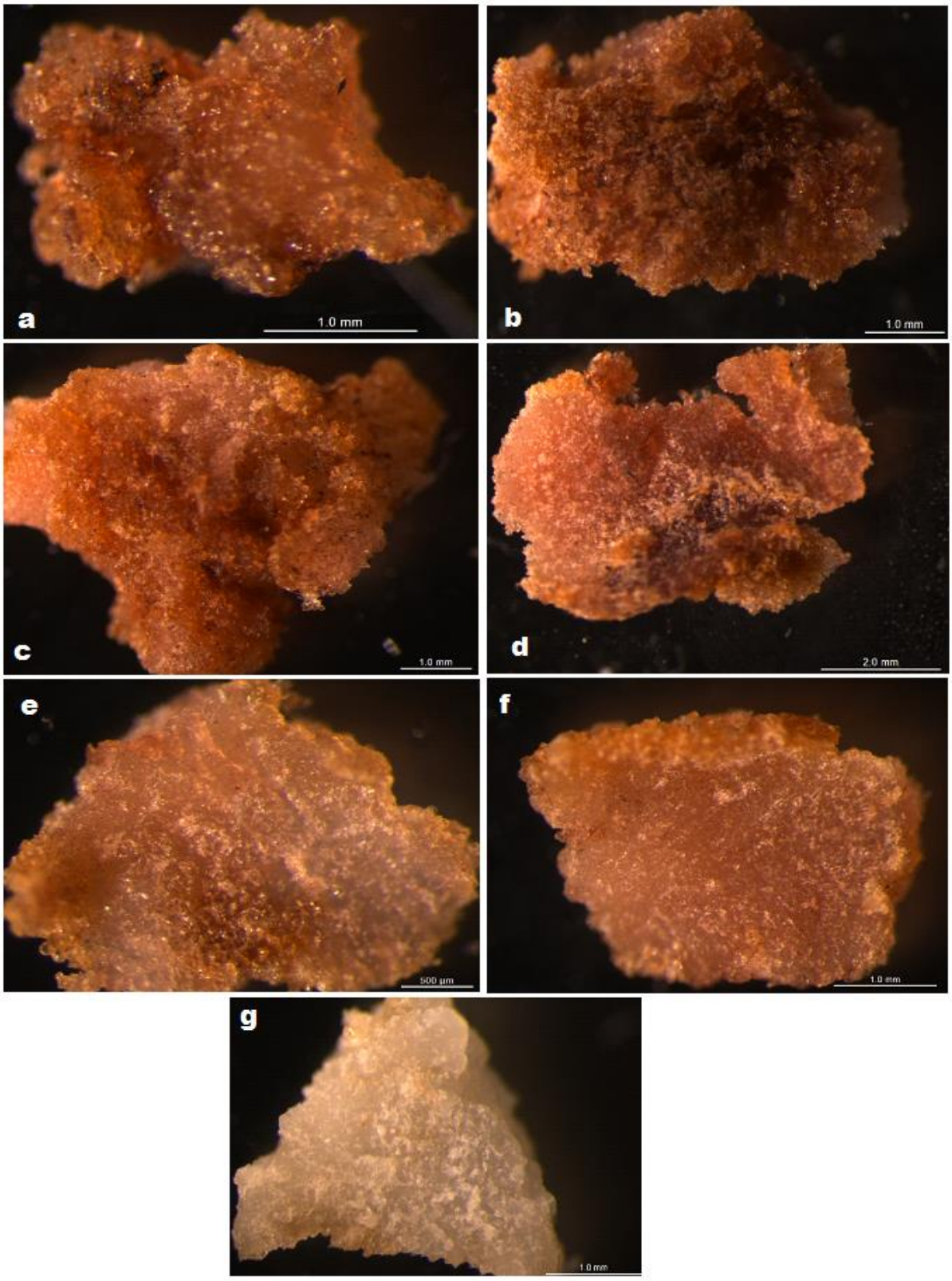


Figure 16. Histochemical staining of H_2O_2 in calli subcultured on 0 (a), 0.005 (b) ,0.05 (c), 0.5 (d), 5 (e) and 50 µM Fe media (f) for three months. Calli were transferred from a medium with optimal (50 µM) Fe medium and subcultured on to the respective Fe-deficient media at 4-weeks intervals. Callus without DAB staining is represented by letter g (negative control).

Production of H_2O_2, was found be influenced by the amount of Fe supplied to callus medium. Exclusion of Fe from medium caused stronger brown colouration throughout calli indicating a wide distribution and/or increased production of H_2O_2.The results of this study reveal that H_2O_2 reactive species production was strongly enhanced under Fe deficiency conditions. This suggests that Fe shortage possibly activated systems responsible for H_2O_2 production. Fe-deficiency conditions induced increased H_2O_2 production evidenced by presence of high amounts of brown precipitates. Formation of H_2O_2 was pronounced in long term cultures suggesting that the enhanced H_2O_2 staining observed in calli after three months of culture may be due to a rise in H_2O_2 generation in response to oxidative stress. Fe-deficiency induced oxidative stress may have been severe due to prolonged exposure to Fe-deficiency stress. Previous studies have reported likewise. Ranieri et al. (2001) recorded increased H_2O_2 contents in sunflower due to iron deficiency. The lack of Fe led to increased H_2O_2 levels in maize (Sun et al., 2007; Tewari et al., 2005) as well as mulberry and cauliflower plants (Tewari et al. 2005). Accordingly, Tewari et al. (2013) and Ramírez et al. (2013) showed an upsurge in ROS production due to Fe deficiency. Iron is a component of electron transport chains in the mitochondria and chloroplasts and therefore iron deficiency can disrupt electron transfer and lead to overproduction of ROS (Ramirez et al., 2013). ROS was detected to be localised primarily in the chloroplasts of iron-deficient Arabidopsis seedlings (Ramirez et al., 2013). Impaired photosystem II efficiency resulted in H_2O_2 accumulation in chloroplasts and higher levels of H_2O_2 was observed in Fe-deficient than in Fe-sufficient plants (Tewari et al., 2005, 2013).

Cells control the levels of ROS by creating equilibrium between ROS elimination and their generation. However, for example, under metal stress, ROS generation increases causing oxidative stress and/or cellular injuries (Rout et al., 2015; Wang et al., 2011). Immoderate levels of ROS result in oxidative stress which can have deleterious effects such as DNA and RNA damage, oxidation of proteins, membrane lipid peroxidation, inactivation of calcium dependent proteolytic enzyme and may eventually result in cell death (Panda et al. 2012; Bhaduri and Fulekar, 2012; Tewari et al., 2013). Excess H_2O_2 production in response to stress in most Fe-deficient and/or long term callus cultures may be accountable for the cellular damage caused via lipid peroxidation and necrosis or cell death observed in callus cultures. Oxidative stress resulting from the formation of ROS causes release of iron from proteins and H_2O_2 is reduced by Fe^{2+} resulting in the formation of hydroxyl radical (Perron and Brumaghim, 2009).

The low levels of H_2O_2 detected in calli supplied with optimal Fe quantity could be as a result of the normal H_2O_2 produced during cellular processes. ROS are usually constantly generated under physiological conditions in living organisms as a consequence of aerobic metabolism and cell signalling mechanisms (Bhaduri and Fulekar, 2012; Rout et al., 2015). ROS are required in only sublethal quantities for normal plant growth since they are highly cytotoxic (Bhaduri and Fulekar, 2012; Rout et al., 2015). The decreased levels of H_2O_2 revealed by DAB staining in some callus cells exposed to Fe deficiency is consistent with the notion that variants in calli tolerant to Fe deficiency were better protected against oxidative damage than Fe-deficiency sensitive cells. This is in agreement with M'sehli et al. (2014) who found that Fe starvation resulted in decreased H_2O_2 accumulation in a tolerant line than a sensitive one. A significant increase in H_2O_2 content was found in the Fe-deprived tissues of chlorosis sensitive peach rootstocks (Molassiotis et al., 2006). The information obtained by histochemical analysis of H_2O_2 in this study may imply that H_2O_2 build-up occurs in calli that experience chlorosis. Modifications of cell wall properties during Fe deficiency stress adaptation can be due to H_2O_2 accumulation in root tissues of chlorotic plants (Donnini et al., 2011).

3.6.3 Fe-deficiency associated with decreased antioxidant enzyme activities

Peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) are heme-containing enzymes and play important roles in the scavenging of ROS produced in plants under stress conditions. Since, iron deficiency is an abiotic stress for plants, it was reasoned that the activities of POD, CAT and APX are likely to be affected in calli cultured under such conditions. POD, CAT and APX activities were evaluated to verify whether variations in iron supplies have an influence on these antioxidant activities.

3.6.3.1 POD enzyme activity

Overall, as shown in the boxplot (Figure 17) POD activity was highest in callus cultures growing on the sufficient Fe medium irrespective of the culture duration. POD activities in calli exposed to 0 and 0.005 µM Fe medium for one month was respectively 3.2 and 2.1 units lower ($p<0.05$) compared to calli on 50 µM medium. POD activities of calli on 0.05 to 5 µM Fe medium were similar (13-12 units). POD activities of calli cultured under Fe-deficiency

stress for three months were lower than those cultured for a month under the same conditions but the reverse occurred in calli growing on the control medium.

After three months, there was a steady increase in POD activity in calli on media containing varying Fe concentrations from 0 to 0.05 µM but there was a lower enzyme activity in calli on 0.5 µM Fe-containing medium. A marked rise in POD activity was found in calli cultured on 50 µM Fe-containing medium. On this medium, POD activity was found to be elevated by about 11% as the culture duration prolonged. The mean POD activities of calli on Fe-deficient (0.005-5 µM) were 22-28% significantly lower than that of calli on medium with sufficient Fe levels. The mean POD activity of calli on Fe sufficient medium was 35.

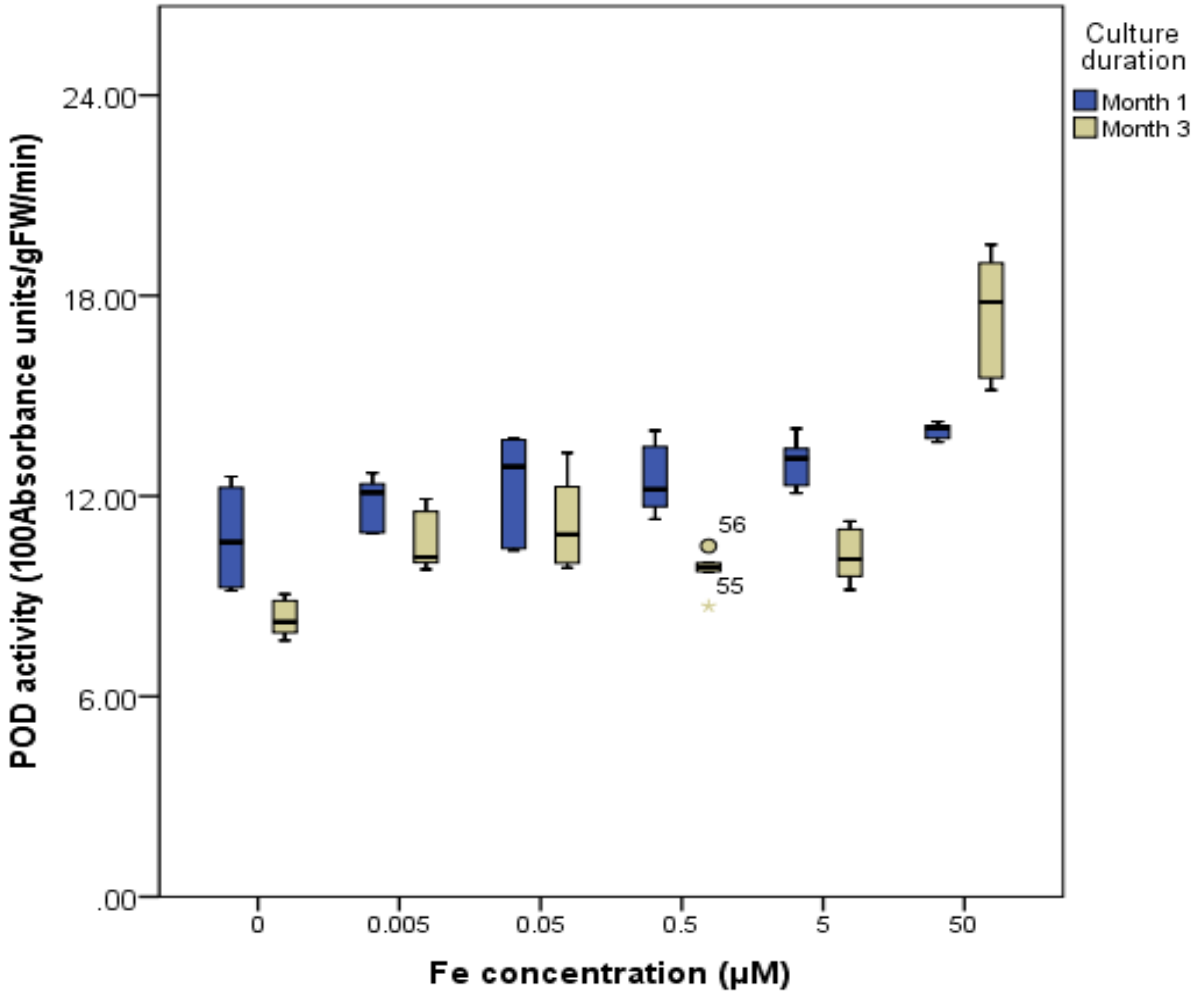


Figure 17. Response of POD activity to Fe deficiency in calli cultured for shorter and prolonged periods. Calli transferred from a sufficient (50 µM) Fe medium to Fe-deficient media (0-5 µM) were assayed for POD activity after one month (month 1) and subsequent two subcultures up to three months (month 3).

3.6.3.2 APX enzyme activity

APX activity in calli was gradually increased with a rise in Fe concentration up to 5 µM in the culture medium but calli on 50 µM Fe-containing medium exhibited a sharp decrease in the enzyme activity (Figure 18). APX activities in some callus cells on low Fe media were similar to that of calli on the control medium as indicated by the maximum and minimum values on the box plot (Figure 18). After one month of culture, the mean APX activities of calli on media with 0.005 - 0.5 µM Fe content were similar to that of calli on control medium. As the duration in culture increased to three months, APX activity was further reduced in calli on 0 and 0.005 µM Fe-containing media. However, calli growing on 0.05 to 5 µM Fe supplies were found to have significantly increased (about 2-folds) APX activity compared to calli exposed to the same medium for one month. Calli on 5 µM Fe medium had the highest APX activities after one and three months in culture. There was a 3 to 4-folds significant rise in APX activity between calli on 5 µM and calli on either Fe-deprived or sufficient Fe medium. There was a statistically significant difference in APX activity based on the culture duration ($t= -2.433$, $df=70$, $p<0.05$).

3.6.3.3 CAT enzyme activity

CAT activity in calli was initially higher under Fe-deficiency compared to sufficient Fe conditions but the reverse was observed as the culture duration was increased as shown by the boxplot in Figure 19. After one month of culture, there was a significantly sharp rise in CAT activity in calli on media containing 0 to 0.005 µM Fe and then a steady increase in the enzyme activity after which a drastic reduction in CAT activity was found in calli on 50 µM Fe medium (see Figure 19). CAT activity in calli on Fe-free medium was comparable ($p>0.05$) to that in calli supplied with sufficient quantity of Fe. However, CAT activities of calli exposed to Fe-deficiency stress for one month was significantly elevated relative to calli with sufficient Fe supplies. After three months, CAT activity was increased in calli on media with an increase in the Fe level in culture medium (Figure 19). Sufficient Fe supplies seemed to influence CAT activity positively over the prolonged culture duration. Calli on 50 µM Fe-containing medium exhibited higher CAT activity after three months than after one month.. The results clearly indicate that the level of Fe in the growth medium affected CAT activity in potato calli.

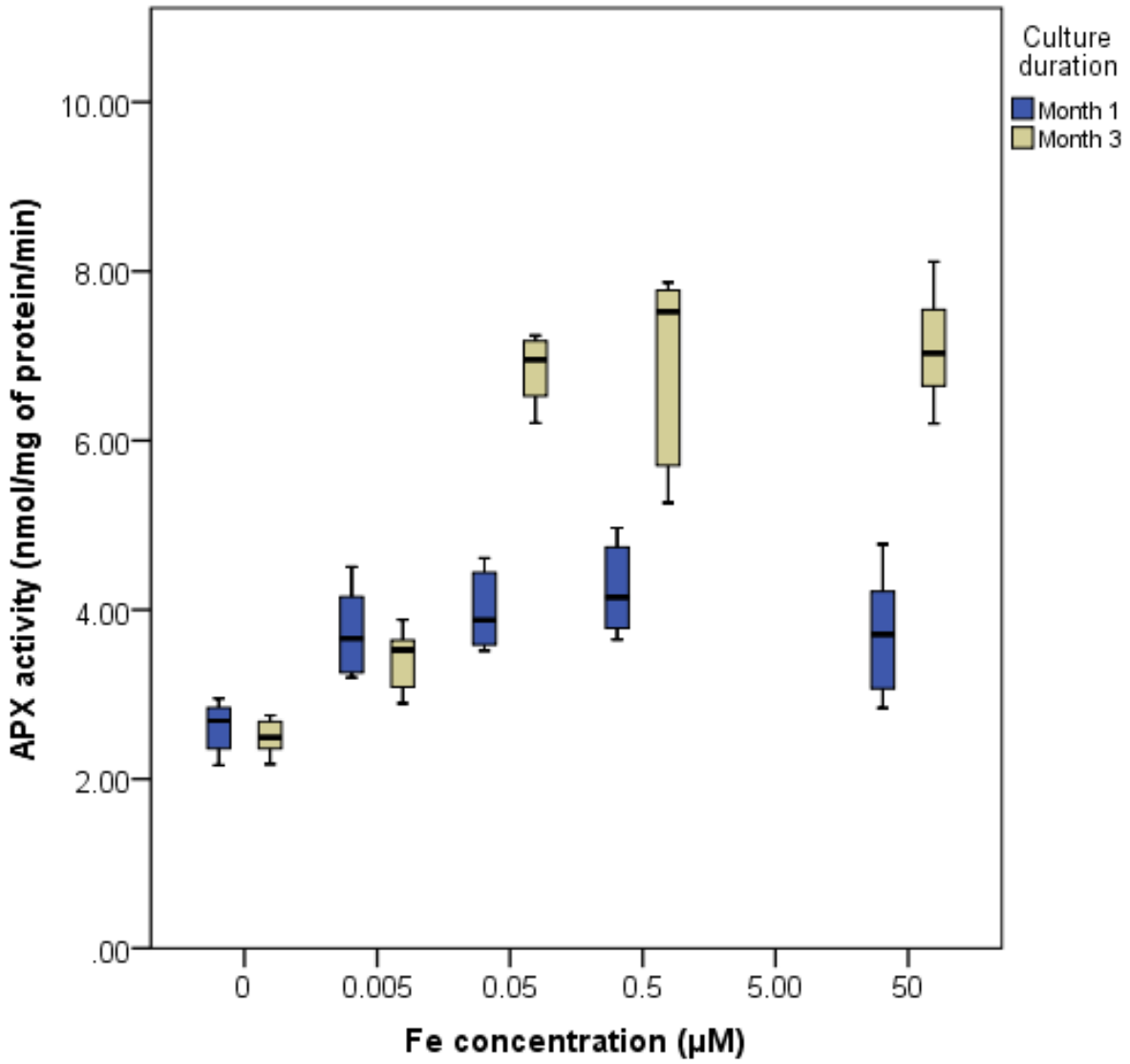


Figure 18. Changes in APX activity to in response to Fe deficiency in calli cultured for shorter and prolonged periods. Calli transferred from a sufficient (50 µM) Fe medium to Fe-deficient media (0-5 µM) were assayed for APX activity after one month (month 1) and subcultures at 4-weeks intervals for up to three months (month 3).

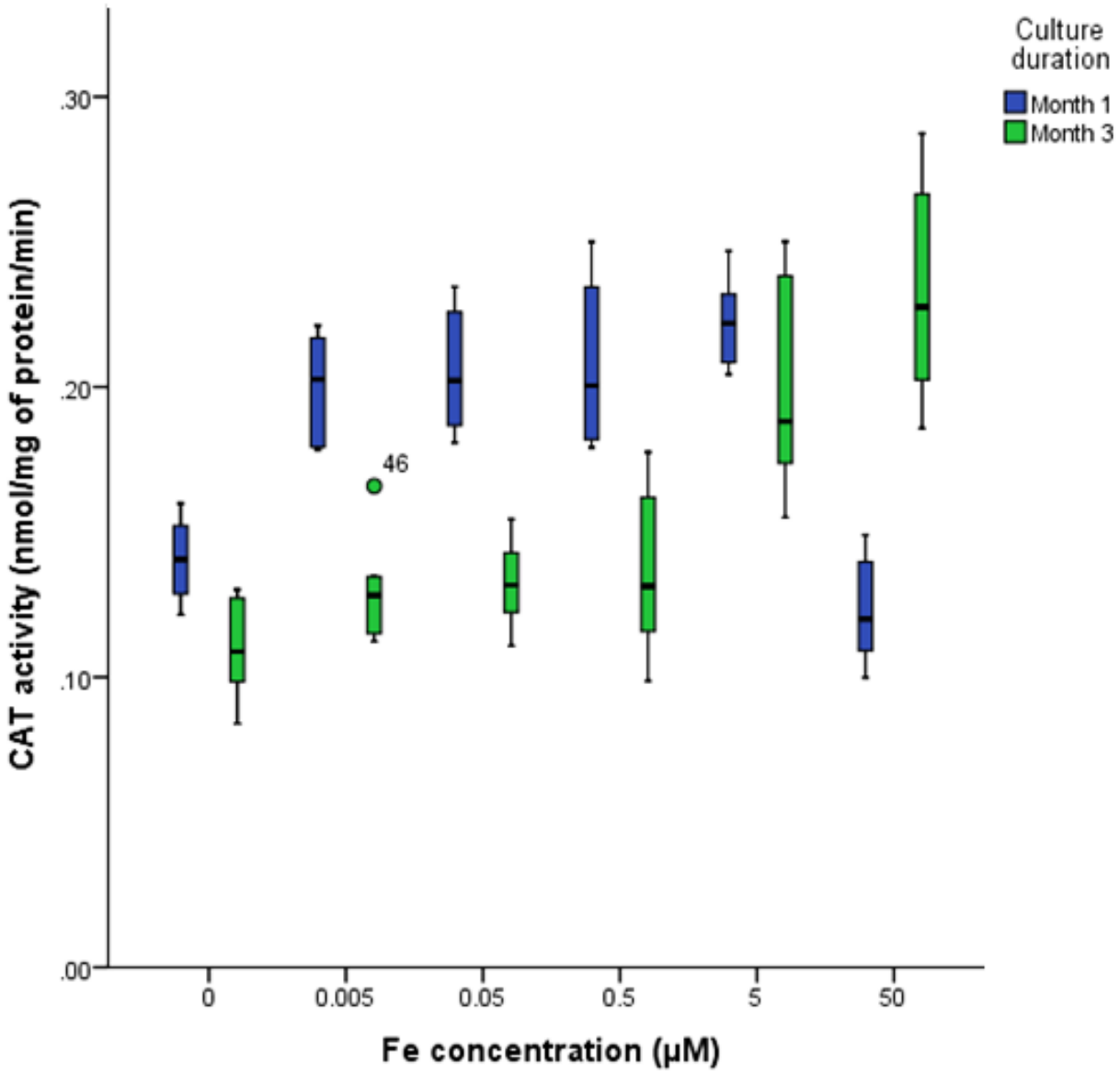


Figure 19. Changes in CAT activity in response to Fe deficiency in calli cultured for shorter and prolonged periods. Calli transferred from a sufficient (50 µM) Fe medium to Fe-deficient media (0-5 µM) were assayed for CAT activity after one month (month 1) and subsequent subcultures at 4-weeks intervals for up to three months (month 3).

As previously noted, POD, CAT and APX play important roles in scavenging ROS and are involved in antioxidative defence response (Zamboni et al., 2012; Rout et al., 2015; Tewari et al., 2013). Guaiacol peroxidases use guaiacol as electron donor and decompose H_2O_2 to H_2O and O_2. Plant cells exposed to Fe deficiency can be sensitive to oxidative stress due to a low level of the antioxidant, peroxidase (Ramírez et al., 2013). In the current study, decreased POD activity under limited Fe supply may have been due to low activation and/or a reduced production of the POD enzyme. POD is a heme-containing enzyme and therefore its activity and/or synthesis are probably affected by iron deficiency. In previous studies, Kabir et al. (2015) detected that POD activity in Okra plants was decreased due to Fe deficiency. Salama et al. (2009) also showed that POD activity decreased significantly in flax cultivars under iron deficiency conditions. Transcripts encoding peroxidase enzyme was found to be down-regulated in tomato leaves under low Fe conditions (Zamboni et al., 2012).

Ascorbate peroxidase (APX: EC 1.11.1.11) is a peroxidase of ubiquitous occurrence in plants and considered as a universal housekeeping protein in plant chloroplasts and cytosol with a heme cofactor (Ramirez et al., 2013). APX scavenges H_2O_2 produced by superoxide dismutase using ascorbate (substrate) as an electron donor (Panda et al., 2012). Similar to the finding of this study, APX activity was low due to exposure to iron deficiency in *A. thaliana* (Ramirez et al., 2013). Calli on the sufficient Fe medium for one month probably did not experience any appreciable stress to cause the cells to initiate antioxidant response via APX activation. Thus, APX levels in calli on control medium were low. Possibly, Fe stores acquired from prior exposure of the calli to sufficient Fe levels may have accounted for more elevated APX activity in them after one month of Fe-deficiency stress conditions compared to calli after three months. After this prolonged time of culture which had repeated exposure to Fe-deficiency, any excess Fe-stores would possibly have been depleted of Fe-stores. It appears that the Fe-deficiency conditions capable of supporting APX activation in aging cultures was 0.5–5 µM Fe. There was a significant rise in APX activity in calli at 5 µM which may imply that 5 µM Fe was adequate to elicit antioxidative enzyme response to Fe-deficiency via an increase in APX activity.

Catalase (CAT: E.C 1.11.1.6) functions in the detoxification of immoderate levels of stress induced ROS in plants. CAT is an oxidoreductase and a tetrahedral protein with four heme groups at the catalytic centres (Boon et al., 2007). It is predominantly localised in the peroxisomes (Bhaduri and Fulekar, 2012; Rout et al., 2015) and catalyses the dismutation of H_2O_2 to H_2O and O_2 in the reaction, $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. It appears that calli on control media were not under considerable stress after one month to stimulate CAT antioxidant

response. Although callus cells cultured in medium lacking Fe may have been stressed, CAT activity was least in these cells probably because CAT activity and/or expression was not favoured under low Fe conditions. The further decline in CAT activity in the calli subjected to extended periods of Fe-deficiency stress (long term cultures) may be associated with insufficient Fe levels. A 5 µM Fe is proposed to be adequate to elicit an elevated CAT antioxidant respose to Fe-deficiency induced stress.

Taken together, calli exhibited reduced antioxidant enzyme activities in response to Fe-deficiency stress conditions as was observed in earlier studies. CAT, POD, and APX activities in mulberry, maize, and cauliflower plants decreased due to exposure to iron deficiency (Tewari et al., 2005). Following iron deficiency, the activities of APX, POD and CAT were considerably reduced in flax cultivars (Salama et al., 2009). The authors suggested a reduced functional Fe status in plants under iron deficiency and hence, decrease in the activities of these iron-containing antioxidative enzymes. Zamboni et al. (2012) reported the down-regulation of mRNA encoding catalase and peroxidase in tomato under Fe deficiency conditions. In response to Fe deficiency, CAT protein levels decreased while that of PODs increased in tomato roots (Brumbarova et al., 2012; Zamboni et al., 2012). Studies on New Zealand-grown potatoes revealed that antioxidant activity was correlated with the phenolic content of the potatoes (Lister and Munro, 2013).

It appears that synthesis and/or activation of POD, CAT and APX antioxidant enzymes is influenced by the amount of Fe in the growth medium. The ample supply and possible enhanced Fe uptake at 50 µM may have facilitated POD, CAT and APX biosynthesis since Fe is a component of these antioxidant enzymes and necessary for their functioning. Although the callus cells experienced oxidative stress during exposure to Fe-deficiency conditions, antioxidant enzyme synthesis may have been low owing to low amounts of Fe in growth medium resulting in the decreased activity levels measured. Fe-deficient calli may have experienced elevated stress as the culture duration increased but this did not translate into increased antioxidant enzyme response. This seems to suggest that a sufficient Fe supply is required to promote and sustain antioxidant response to stress conditions. It appears that a minimal amount of Fe (5 µM) is required to elicit APX and CAT antioxidant response to Fe-deficiency induced oxidative stress. It has been proposed that plants may be highly sensitive to oxidative stress following Fe starvation because Fe is a component of the antioxidant enzymes (Kumar et al., 2010; Ramirez et al., 2013).

Calli on sufficient Fe medium showed elevated POD and CAT activity after two subcultures probably due to stress associated with *in vitro* culture. After three months of

culture *in vitro*, all irrespective of the Fe content in culture medium, long-term subculturing of calli can cause calli to undergo appreciable stress. This tissue culture-induced stress may have elicited antioxidant response in terms of POD and CAT activities to magnitudes highest in calli growing on sufficient Fe medium. Similarly, calli growing on control medium over a prolonged period in culture showed elevated APX activity response. This is in consonance with the previous finding that culture conditions and periods can cause stress to the cells in culture. Culturing cells *in vitro* can impose some stress and mutations in cells (Phillips et al., 1994; Bairu et al., 2011) and the effect of the stress seems to be pronounced in long term cultures as shown in this study.

Antioxidant enzyme activity appears to be independent of H_2O_2 production under Fe-deficiency conditions. It has been discovered in this study that Fe-deficiency stress induced H_2O_2 but does not seem to cause a commensurate elevated antioxidant response (APX, CAT and POD activities) for H_2O_2 scavenging. During redox homeostasis, the antioxidant system maintains a balance between production and scavenging of ROS; such a balance is critical for the protection of the system against oxidative burst. However, the lack of regulation of ROS normally leads to oxidative stress which can have lethal effects on cells. This may explain why most cells cultured for prolonged periods under Fe-deficiency conditions develop necrosis and/or die. The increased level of H_2O_2 may be the result of insufficient activation of heme-containing POD and CAT, due to low Fe availability. The decreased activities of antioxidant enzymes under iron deficiency conditions suggest that they may not be essential in the detoxification of ROS (Sun et al., 2007).

3.7 Response pattern recognition with principal component (PCA) and cluster analysis (CA)

PCA and CA are pattern discovery techniques and are a focussed type of data mining techniques used to detect different patterns in large datasets. They are used as a means of measuring the degree of similarity among variables. PCA and CA aid in organising observed data groups in a way such that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise, without information about the variables involved in the group separation or about their relative importance (Conti et al., 2009). These techniques can be used to identify what patterns among variables might be embedded in the datasets.

PCA and CA were used to provide an initial indication of the number of factors that may be contributing to the response of calli to Fe supply in relation to the duration in culture. Topology of the iron status of the media in which calli were grown and the duration of exposure to these media was built using a set of data which gives a picture of the profile of calli. Pattern recognition and data reduction analysis of the morphological and biochemical responses of calli to Fe supply were carried out using data from the various biochemical assays and fresh weight measurements. The factor was also employed to gain understanding of the differences in relationships of the morphological and biochemical response parameters with other varibles (Fe concentrations in growth medium) not used to build the clusters. The cumulative percentage of variance explained by the PCA as well as the similarity of the pattern-recognition outputs with the results from CA were used to evaluate their reliability. The closeness of the outputs from PCA and CA on the same dataset is a model fit indicator.

Grouping of calli based on morphological (fresh weight) and biochemical measurements gave an indication of similar patterns of responses of calli to differences in iron supply and the duration of exposure to variations in Fe nutrition (Figure 20). Classification of the morphological and biochemical characteristics of calli exposed to different treatments using PCA and CA yielded three principal components (with Eigenvalues >1) and three clusters (see Appendix, Figure B2) respectively. The PCA model explained 67.27% of the total variance in the dataset. Details of the communalities of the dataset and the rotated component matrix showing factor loadings are provided in Appendix (Table B3). Variables grouped under each principal component may share related biochemical characteristics based on response to Fe-status of growth medium.

In the first month of exposing calli to different Fe supplies, calli on Fe-sufficient medium were found to be linked to factors such as total chlorophyll and carotenoid contents, POD activity and fresh weight .These characteristics were similar in that they were highly elevated in calli with sufficient Fe nutrients and such significantly increased responses occurred in calli over a one month culture. Similarly, using PC analysis, Tewari et al. (2013) grouped chlorophyll content, CAT, iron content and TBARS levels in *Brassica napus* leaves as factors linked to Fe-sufficiency conditions. FCR and CAT enzyme activities, TBAR and total phenolics levels were enhanced in calli under Fe-deficiency conditions and are factors linked to the responses of calli exposed to no or low Fe nutrition for a month.

Overall, PC membership is heavily impacted by the Fe content in the callus culture medium. The Fe status of media were assigned to the different response factors and clusters based on the rotated varimax value for each component and the data of the individual tests

carried out. Calli under Fe-deficient (0.005-5µM) conditions of growth were moderately affected by the parameter sources in the data. Calli on Fe-deficient media mostly exhibited minimal to average response with respect to the morphological and or/biochemical parameters assessed and were therefore placed midrange on the PCA plot (Figure 20).

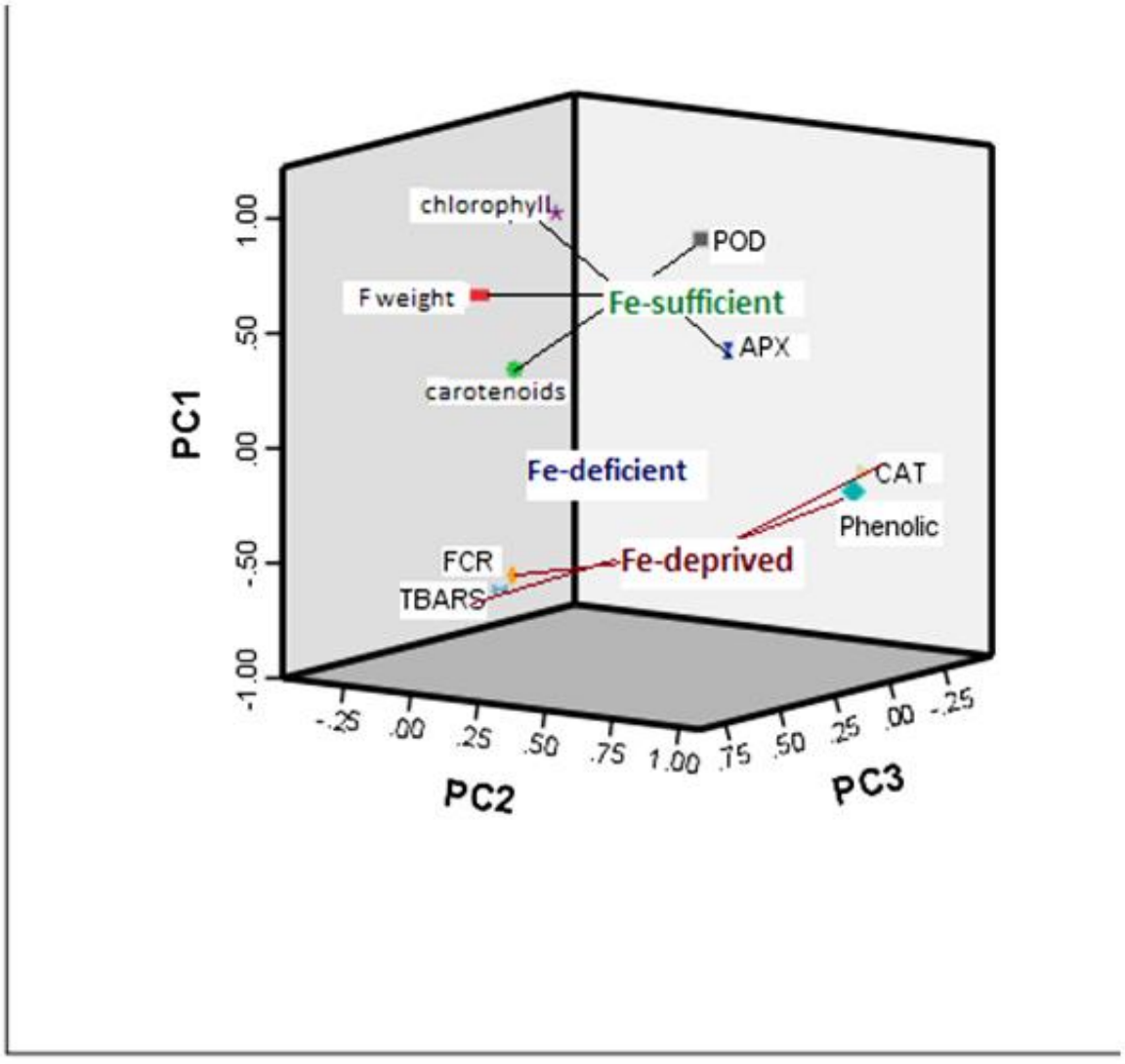


Figure 20. Principal component analysis of factors that characterise variable iron supply in calli exposed to Fe-deprived (0 µM), Fe-deficient (0.005-5 µM) and Fe-sufficient (50 µM) media for a month.

The classification of calli based on the fresh weight and biochemical parameters measured in calli cultured under Fe-deficient and Fe-sufficient conditions for three months is shown in Figure 21. The analysis resulted in two principal components (PCs) with Eigenvalues >1. Overall, the PCA model explained 65.6% of the total variance in the data. Communalities of all the parameters considered are up to 81%. The first principal component (PC1) consists of APX, CAT, POD, Chlorophyll, carotenoid, TBARS and fresh weight parameters whereas PC2 is made of phenolic content and FCR activity. The rotated component matrix showing factor loadings is provided in (see Appendix Table B4). The PCA grouping of the parameters was quite similar to the grouping generated by CA (see Appendix Figure B3).

On the whole, the parameters within a principal component are interpreted as the biochemical responses of calli exposed to a similar set of conditions (i.e. Fe concentration in medium). Factor loading plot of PC1 versus PC2 shows which component is associated with calli exposed to either Fe-deficient or Fe-sufficient growth conditions (see Figure 21). PC1 comprise factors which show similar response patterns in that they were found to be significantly high in calli maintained on Fe-sufficient medium for three months of subcultures. PC2 membership was assigned to calli exposed to Fe-deprived medium for three months since such calli were detected to have a similar response of increased phenolic content and FCR activity which is not a characteristic identified with calli on Fe-sufficient medium. Figure 21 shows that Fe-deprived calli is clearly differentiated from the Fe-sufficient ones indicating a distinction in their morphological and biochemical response patterns to Fe supply after three months in culture.

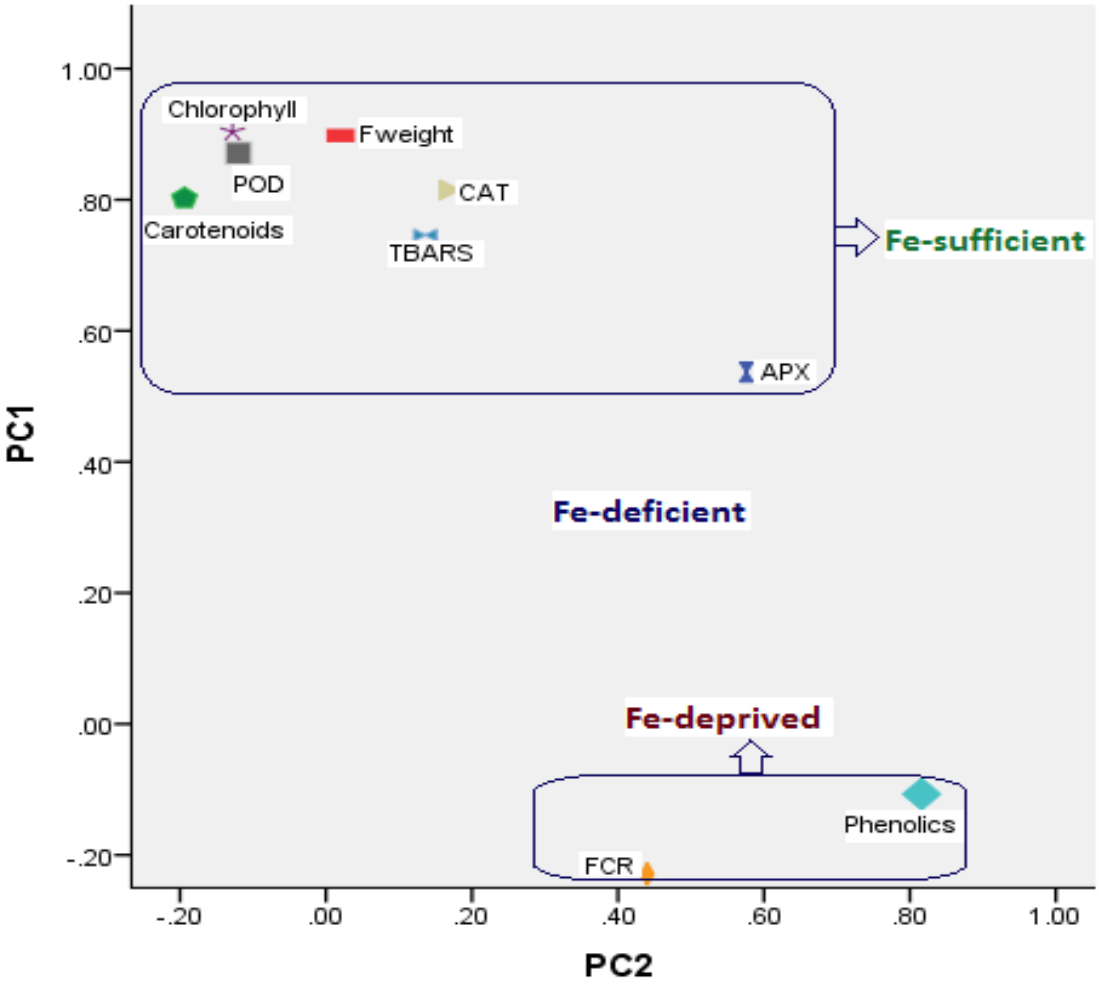


Figure 21. Principal component analysis of factors that characterise variable iron supply in calli exposed to Fe-deprived (0 µM), Fe-deficient (0.005-5 µM) and Fe-sufficient (50 µM) media for three months.

Clasifications of the morphological and biochemical characteristics of calli in response to Fe nutrition is analogous to that obtained from the PCA output.The CA dendogram generated three and two clusters for month one and three calli cultures respectively (see Appendix Figures B2 and B3). CA uses all the variance in a data set and the Ward's method was selected for the CA model because it possesses a small space distortion effect, uses more information on cluster contents than other methods, and has been proved to be extremely powerful grouping mechanism (Aničić, et al., 2009). The parameters chlorophyll and carotenoid content, fresh weight and POD activity belonged to one cluster while phenolic content, TBARS levels, FCR and APX activities were linked to the other clusters. Cluster membership has some implication for predicting similarities. CA has the

capability to uncover the intrinsic structure or underlying behaviour of the dataset, without making *priori* assumptions about the data, in order to classify the objects of the system into categories or clusters based on their nearness or similarity (Vega et al., 1998).

3.8 Test for relationships between responses of calli to Fe supply

In order to test the hypothesis that the amount of Fe available to calli influences the biochemical and morphological responses generated, the correlation between each measured variable in calli was investigated. Correlation analysis was used to test relationships between the variables. Spearman's rho and Pearson *r* were used to explore the correlation between relevant variables within the datasets. The appropriate method applied for the relevant datasets were selected based on the outcome of the normality tests. In this section, the correlation between each studied variable was evaluated in calli grown for different durations in culture under different Fe treatments. Information on this relationship will broaden our understanding of the effect of Fe concentrations and culture duration on calli. The correlation analysis can provide answers to whether there are significant relationships between the tested morphological and biochemical variables in calli. The analysis can also be used to find out if Fe concentration in callus medium correlate with the responses exhibited.

The correlation analysis (see Table 5) indicates that CAT activity, carotenoid and total phenolic content were weakly related to high Fe supplies in the first month of culturing calli. Within one month, significantly positive correlations were found between Fe levels and APX, POD enzyme activities as well as total chlorophyll content and fresh weight. An increase in Fe concentration positively affected antioxidant enzyme (APX, POD) activities and chlorophyll content. The activity of FCR, the enzyme which plays an important role in Fe uptake in Strategy 1 plants like potato, was strongly correlated in a negative manner with POD activity, Fe concentration and total chlorophyll content. In contrast, FCR activity and TBARS levels in calli were significantly related in a positive manner. The correlations among Fe concentration, FCR activity and lipid peroxidation (TBARS levels) were significantly negative; an indication that at sufficient Fe levels, FCR activity was not enhanced ($r = -0.42$; $p<0.05$) and TBARS level was considerably minimal ($r = -0.73$; $p<0.01$). This implies that under conditions of reduced Fe supply for a month, FCR activity was elevated while there were reduced chlorophyll content coupled with low POD activity and high levels of TBARS.

Chlorophyll content increased significantly with a rise in the amount of Fe available to calli. Calli with high chlorophyll content had a markedly decreased FCR activity ($r = -0.45$; $p<0.01$), TBARS levels ($r = -0.62$; $p<0.01$) and elevated POD activity ($r = 0.49$; $p<0.01$). Total phenolic content displayed a significantly positive relationship with APX and CAT activities implying that these antioxidant enzyme activities were highly stimulated when the phenolic content in calli was increased. Although the mean total phenolic content in calli cultured for one month was reduced significantly with an increase in Fe levels in medium (see Figure 21), total phenolic content was weakly related to Fe concentration ($r = -0.10$; $p>0.05$). Similarly, total phenolic content showed a weak relationship with POD activity ($r = -0.13$), FCR ($r = 0.04$), chlorophyll ($r = -0.10$), carotenoid ($r = 0.03$) TBARS levels ($r =-0.05$) and fresh weight ($r = -0.19$). Weak correlations were found among callus fresh weight and phenolic content, TBARs levels, FCR and APX enzyme activities. The results show that the weight of callus was not related to CAT activity. With respect to the relationship between morphological and biochemical responses of calli after one month of culture, there was a significantly positive correlation among callus fresh weight and Fe concentration, POD activity, total chlorophyll and carotenoid contents (Table 5). This indicates that weight increase was related to increases in Fe concentration, POD activity as well as total chlorophyll and carotenoid content.

The correlation analysis of the morphological and biochemical variables associated with callus growth and Fe content in medium after three months is summarised in Table 6. There is a significantly positive correlation between Fe concentration with APX, POD, CAT activities, TBARS levels, total chlorophyll and carotenoid contents in calli subcultured for three months. The accumulation of Fe in calli and the effect on growth may be evidenced by the highly positive correlation of Fe content with chlorophyll contents ($r = 0.68$; $p<0.01$) and callus fresh weight ($r =0.82$; $p<0.01$). Weight gain was strongly related to increased Fe content in growth medium and total chlorophyll content for calli subcultured for three months. Furthermore, callus morphology (fresh weight) showed a significantly positive correlation with biochemical parameters such as APX, POD, CAT activities, carotenoid content and lipid peroxidation (TBARS levels). FCR activity and phenolic contents were found to have a simple and weak relationship with callus weight and the other biochemical parameters measured in calli cultured for three months under varying Fe treatments. Over a prolonged period in culture, FCR activity diminished probably due to adaptive mechanisms and this is evidenced by the weak and mostly negative relationship between FCR activity and other biochemical responses of calli to Fe supply.

From the results (Table 6) it appears that weight increase as culture duration increased and these were related to high TBARS levels, phenolic, chlorophyll and carotenoid contents together with elevated antioxidant activities. The effect of prolonged culture duration and the potential increased production of ROS in calli under *in vitro* conditions is depicted by the strong positive correlation between Fe concentration with lipid peroxidation, APX, POD and CAT activities. This gives an indication that after two subcultures, increasing Fe supplies was strongly related to increased lipid peroxidation and enhanced antioxidant activities. Also, lipid peroxidation was closely related to chlorophyll and carotenoid content, callus weight and Fe concentration.

Some response variables measured in calli differed across culture periods (one and three months). The correlation between Fe concentration and carotenoid content in calli after one month of culture was weak ($r = 0.25$; $p>0.05$) but a stronger relationship ($r = 0.61$; $p<0.01$) was recoded after three months of culture. Correlation between Fe concentration and phenolic content was slightly negative ($r = -0.10$; $p>0.05$) after calli were cultured for one month but positive after three months ($r = 0.13$; $p>0.05$). Also, there was a significantly negative relationship ($r = -0.73$; $p<0.01$) between TBARS levels and Fe concentration in calli after culture for one month but positive after three months ($r = 0.63$; $p<0.01$). The correlation between FCR activity and chlorophyll content in calli was significantly negative after culture for one month ($r = -0.45$; $p<0.01$) but it was not significant after three months ($r = -0.07$; $p>0.05$).

Table 5. Spearman's correlation coefficients for biochemical and morphological measurements of calli exposed to varying Fe concentrations for one month (A) and three months (B).

	Fe concentration	APX activity	POD activity	CAT activity	FCR activity	Total Chlorophyll	Carotenoids	Phenolics	TBARS levels	Fresh weight
Fe concentration	1.0	**.33***	**.70****	.01	**-.42***	**.89****	.25	-.10	**-.73****	**.56****
APX activity	**.33***	1.0	.12	**.69****	-.13	.21	**.30***	**.41***	-.15	.25
POD activity	**.70****	.12	1.0	.11	**-.41***	**.49****	.13	-.13	**-.38***	**.42***
CAT activity	.01	**.69****	.11	1.0	.17	-.13	.13	**.65****	.08	.00
FCR activity	**-.42***	-.13	**-.41***	.17	1.0	**-.45****	-.12	.04	**.35***	-.07
Total Chlorophyll	**.89****	.21	**.49****	-.13	**-.45****	1.0	.29	-.10	**-.62****	**.41***
Carotenoids	.25	**.30***	.13	.13	-.12	.29	1.0	.03	-.03	**.37***
Phenolics	-.10	**.41***	-.13	**.65****	.04	-.10	.02	1.0	.05	-.19
TBARS levels	**-.73****	-.15	**-.38***	.08	**.35***	**-.62****	-.03	.05	1.0	-.24
Fresh weight	**.56****	.25	**.42***	.00	-.07	**.41***	**.37***	-.19	-.24	1.0

Table 5B.

	APX activity	POD activity	CAT activity	FCR activity	Total chlorophyll	Carote-noids	Total phenolics	TBARS levels	Fresh weight	Fe concen-tration
APX activity	1.0	**.34***	**.51****	.04	**.38***	**.36***	.27	**.41***	**.59****	**.78****
POD activity	**.34***	1.0	**.52****	-.11	**.48****	**.48****	.15	**.46****	**.52****	**.60****
CAT activity	**.51****	.52**	1.0	-.07	**.57****	**.46****	.11	**.51****	**.69****	**.80****
FCR activity	.04	-.11	-.07	1.0	-.07	-.16	.13	-.10	-.23	-.09
Total chlorophyll	**.38***	**.44****	**.57****	-.07	1.0	**.87****	-.09	**.57****	**.56****	**.68****
Carotenoids	**.36***	**.48****	**.46****	-.16	**.87****	1.0	-.19	**.47****	**.61****	**.61****
Total phenolics	.27	.15	.11	.13	-.09	-.19	1.0	.12	-.03	.13
TBARS levels	**.41***	**.46****	**.51****	-.10	**.57****	**.47****	.12	1.0	**.47****	**.63****
Fresh weight	**.59****	**.52****	**.69****	-.23	**.56****	**.61****	-.03	**.47****	1.0	**.82****
Fe concentration	**.78****	**.60****	**.80****	-.09	**.68****	**.61****	.13	**.69****	**.82****	1.0

**. Correlation is significant at the 0.01 level
*. Correlation is significant at the 0.05 level

3.9 Is there an association between exposure to Fe deficiency and responses?

Using binary logistic regression analysis, the Exp(B) coefficient, Odds Ratio (OR), was employed as a measure of association between the exposure of calli to Fe-deficiency conditions and outcomes or responses (morphological and biochemical). The Exp(B) value (OR) represents the odds that an outcome will occur given a specific exposure (Fe-deficiency), compared to the likelihood of the outcome occurring in the absence of that exposure (Fe-sufficiency or control). Binary logistic regression analysis was carried out to quantify how the absence or low Fe in growth medium was associated with morphological and/or biochemical outcomes or responses in calli. The OR represents the factor by which the likelihood of observing a "Yes" (or a 1) for the dependent variable is increased for each 1-unit increase in the independent variable. If OR > 1, then exposure to Fe-deficiency is considered to be associated with having morphological and/or biochemical outcomes in the sense that having these outcomes raises the likelihood of a calli being exposed to Fe-deficiency conditions. An OR equivalent to 1 (OR=1) implies that exposure does not affect the likelihood of an outcome while if OR<1, then the exposure is associated with lower odds of outcome. Logistic regression was performed in order to assess the following;

a) Whether and which response variables (outcomes) were associated with exposure of calli to Fe-deficiency.
b) What directions (positive, negative, none) of associations existed between the exposure of calli to Fe-deficiency and the response variables measured?
c) Was there an association between Fe concentration and the likelihood that calli on Fe-deficient medium would exhibit a differential response to calli on sufficient Fe medium?

Generally, odds ratios for FCR, CAT, total phenolics and TBARs levels in the initial month of subcultures, were significantly > 1 (Table 7). This implies that Fe-deficiency was highly associated with having these biochemical responses such that one month's exposure of calli to Fe-deficiency conditions raises the likelihood of having these biochemical outcomes. There was a strong positive association (OR = 1.16×10^5, p = 0.045) between Fe-deficiency and the likelihood of developing a typical Fe-efficiency response, high FCR activity. Exposure to Fe-deficiency conditions was significantly associated with CAT activity implying that a 1-unit increase in CAT activity increased the likelihood of calli being in Fe-deficiency conditions by a factor of 2.34×10^{36}. A positive significant association occurs

between calli growing under Fe-deficiency conditions for a month and the likelihood of excreting phenolic compounds (OR = 42.6, p = 0.043). Specifically, each one-unit increase in phenolic content was associated with an increase in the odds of calli growing under Fe-deficiency conditions by a factor of 42.60. A significant association was found between Fe-deficiency and the likelihood of lipid peroxidation (OR = 17.6, p = 0.029). TBARS level was 17.6-folds likely to be associated with calli exposed to Fe-deficiency conditions for a month.

Other biochemical responses were either of nil or low associations with exposure to Fe-deficiency in one month old of callus culture. The results indicate that OR for APX=1 suggesting that exposure of calli to Fe-deficiency conditions for a month did not affect likelihood of the outcome of elicitation of APX activity (OR = 1.088, p = 0.614). The null hypothesis that APX activity levels in calli exposed to Fe-deficiency and Fe-sufficient conditions in month 1 are similar can be accepted. A significant association was found between Fe-deficiency and the likelihood of a deactivation (decreased) POD activity (OR = 0.01, p = 0.046). The odds of calli exposed for a month to Fe-deficiency was associated with a lower likelihood (0.006 times) of POD activity as an outcome or response. There was also a low likelihood for calli on Fe-deficient medium to be associated with a unit increase in total chlorophyll content (OR = 2.54×10^{-4}, p = 0.183). Similarly, exposure to Fe-deficiency was associated with lower odds of a response in a unit rise in carotenoid content although the association was positive (OR = 0.15, p = 0.583). The relationship, however, was not statistically significant. Therefore, the null hypothesis that carotenoid content in Fe-deficient and sufficient calli did not differ can be accepted.

Exposure to Fe-deficiency was associated with low morphological response in the first subculture. There was a significant association between Fe-deficiency and a reduced likelihood of weight gained (OR = 0.01, p = 0.021). A unit increase in fresh weight was related to the odds of calli growing on Fe-deficient medium by 1.3%. This gives credence to the fact that calli gain a minimal increase in weight in the initial subculture onto Fe-deficiency. Although area of callus growth on Fe-deficient medium was small compared to the control, growth was not totally inhibited. Also, calli on 5 µM Fe recorded significantly higher fresh weights than calli on 0 µM Fe (Figure 7). This may have accounted for the slight increase in weight under conditions of low-Fe availability.

Table 7. Exp(B), Odds ratio, values of the biochemical and morphological responses of calli exposed to Fe-deficiency conditions for a culture duration of one month and after 2 subcultures (three months).

Variable	month one		month three	
	Exp(B)	Sig.	Exp(B)	Sig.
FCR	1.16E+05	0.045	8.21E+04	0.441
CAT	2.34E+36	0.021	1.26E-19	0.005
POD	5.97E-03	0.046	3.86E-08	0.994
APX	1.088	0.614	0.873	0.413
Total phenolics	42.61	0.043	1.977	0.193
Total Chlorophyll	2.54E-04	0.183	2.19E-07	0.996
Carotenoids	0.147	0.583	3.49E-21	0.087
TBARs levels	17.62	0.029	9.49E-05	0.023
weight	1.32E-02	0.021	7.85E-03	0.116

Following three months' successive subcultures of calli on Fe-deficient media, about 60% of the biochemical variables tested were of low association with exposure to Fe-deficiency (Table 7). The likelihood of an association between Fe-deficiency status of the callus medium and responses in CAT, POD, chlorophyll, carotenoid and TBARs levels was limited. The low association measured was only statistically significant ($p<0.05$) for TBARs levels and CAT activity responses to Fe-deficiency (Table 8). Fe-deficiency condition was positively associated with the total phenolic content and FCR activity responses of calli. Explicitly, a one-unit rise in phenolic content and FCR activity was associated with an increase in the likelihood of calli growing under Fe-deficiency conditions by 1.98 and $8.21x10^4$ times respectively. Unlike in culture after one month, the association were not statistically significant. Similar to culture after one month, Fe-deficiency was associated with low morphological response after two subcultures. Fresh weight value had a weak association (OR= 0.01, p= 0.116) to calli under Fe-deficiency conditions for three months (Table 8). This supports the results in Figure 7 which shows that repeated exposure to Fe-deficiency caused a marked decrease in callus weight.

3.10 Fe supply to callus cultures influenced cellular distribution of Fe

Visualisation of Fe localisation and distribution in plants has contributed to improving the understanding of Fe homeostasis in plant cells. Perls staining method was employed to investigate cellular distribution and localisation of Fe in calli exposed to varying Fe supplies. Perls staining technique is sensitive and specific for Fe. It is based on the conversion of ferrocyanide to insoluble crystals in the presence of Fe under acidic conditions. Fe deposits in the tissues stain blue when Fe reacts with potassium ferrocyanide (Perls reagent) to form an insoluble pigment referred to as Prussian blue. Due to the insoluble characteristic of the pigment formed, the blue colour is stable after staining and represents an accurate manifestation of iron localisation in living tissue. Staining of Fe on histological sections is a modification that has alleviated the problem of dye penetration in tissues and thus dramatically improved the resolution of images of Fe localisation in cells (Roschzttardtz et al., 2013). The disadvantage of the *in situ* staining however is the potential for Fe loosely bound to soluble ligands to be washed away and be lost during the fixation and dehydration steps (Roschzttardtz et al., 2011a).

Fe was found to be located mainly within intercellular regions or junctions and in small spherical actively dividing callus cells (Figures 22 and 23). Cellular iron distribution and localisation appeared to be influenced by the amount of Fe available to callus cells. The results show that Fe was widely distributed in calli growing on medium with sufficient Fe levels. In calli supplied with sufficient Fe quantities, there was a build-up of Prussian blue precipitates and the intensity of the stains was high in such calli compared to others (Figures 22 E1 and 23 E3). Fe appears to be most probably associated with cell membranes or nucleus of actively dividing cells (see Figure 24). There were considerably fewer stained cells in microtome sections of calli exposed to low Fe compared to calli on sufficient Fe medium. Some calli exposed to Fe-deficiency conditions showed intense Fe staining (e.g. Figure 22 B1 and 23 A3) and may represent sections of the callus cells (somaclonal variants) with Fe-use efficiency.

Internal structure of the callus tissue as revealed by light microscopy was extremely heterogeneous ranging from small to big and giant cells (Figure 22 G). The calli (compact in nature) consisted of closely packed cells of varying shapes and tracheid-like cells. The xylem vessel cells looked zigzag striped whiles parenchyma-like cells were oval and giant cells (Figure 22 G). The shape of the cells within the callus tissue ranged from spherical to markedly elongated and cuboidal. Large elongated cells appeared to be non-dividing cells

whereas the small spherical cells were actively dividing and growing cells. In potato callus, the small densely packed actively dividing cells were found present in the central region of the callus tissue while giant elongated parenchyma-like cells occurred in the region growing or extending outwards (Figure 23 G). Numerous small spherical structures and actively dividing cells were detected in calli cultured on sufficient Fe medium (Figures 22 and 23). With calli exposed to Fe-deficiency, a large proportion of the spherical cells and structures were observed in calli on 5 µM Fe containing medium compared to Fe-deprived (0 µM) calli. The I_2-KI staining of callus tissue showed the presence of several starch granules (spherical) deposited within the central sections of callus tissues cultured on sufficient Fe medium. (Figure 24). Calli cultured on Fe deficient medium contained less starch granules in their tissues relative to calli supplied with sufficient Fe levels.

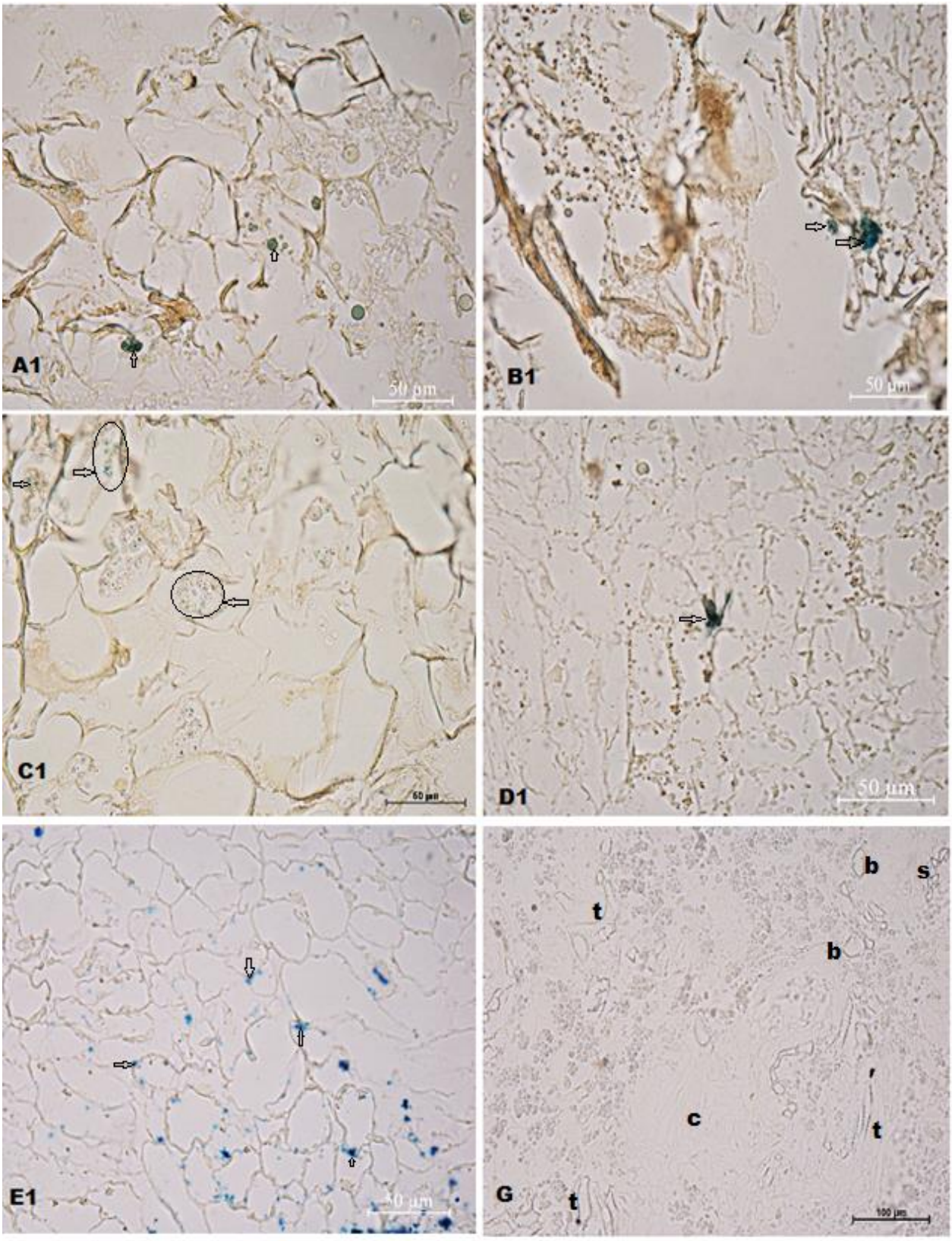


Figure 22. Perls staining of transverse sections of 1-month-old potato callus cultures on Fe-deficient (A1-D1: 0,0.005,0.5 and 5 µM respectively) and sufficient Fe (E1; 50 µM) medium. G is staining negative controls without Perls stain. Blue precipitates of Perls stains represent cellular localisation of Fe (arrow). Different cell types and structures are indicated with letters: b; big cells; c: elongated cuboidal cells, s; small cells, t; tracheid-like cells.

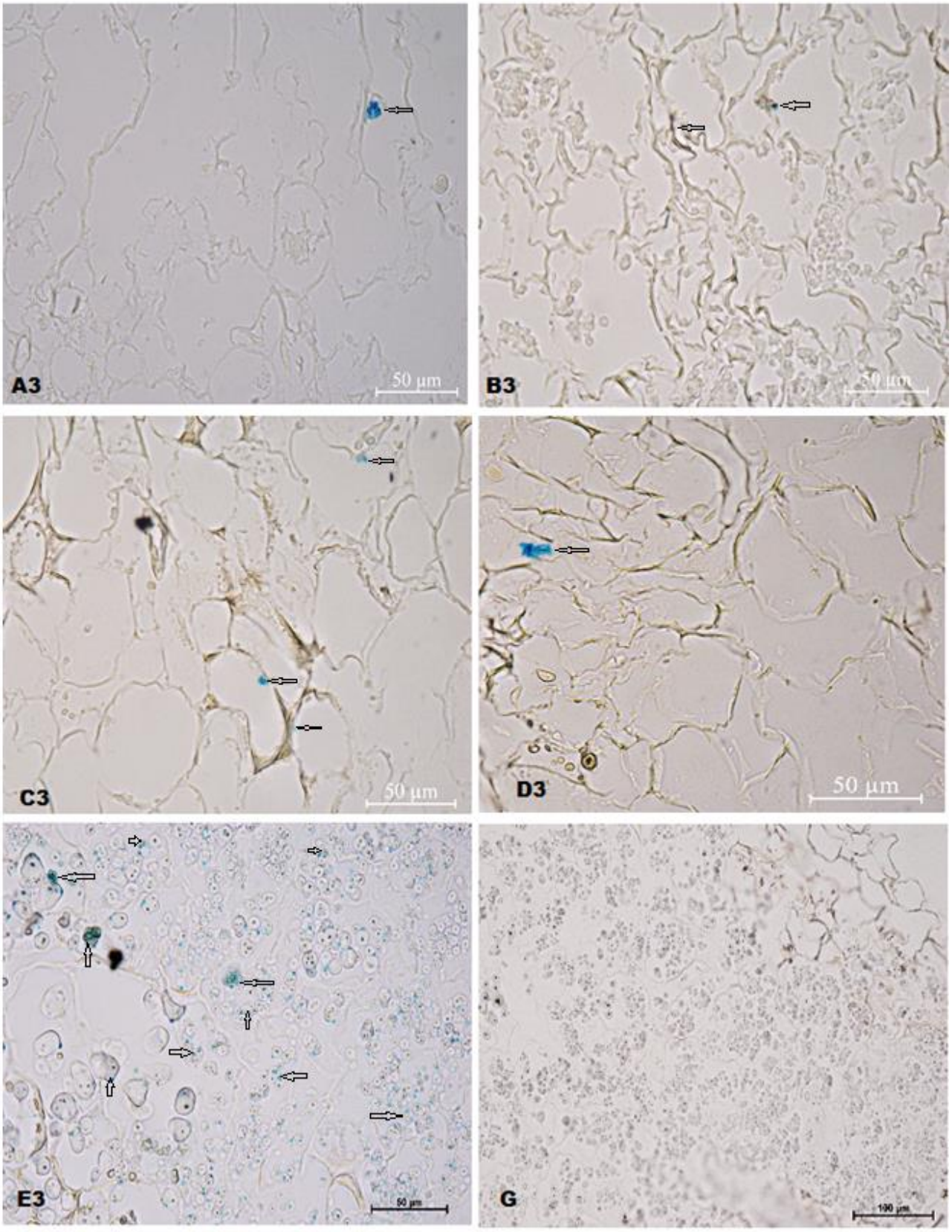


Figure 23. Perls staining of transverse sections of potato calli exposed Fe-deficient (A3-D3; 0,0.005,0.5, 5 µM) and sufficient Fe (E3; 50 µM) medium for three months of subcultures. G: negative control without Perls stain. Arrows indicate some Fe stained (Prussian blue precipitates) regions.

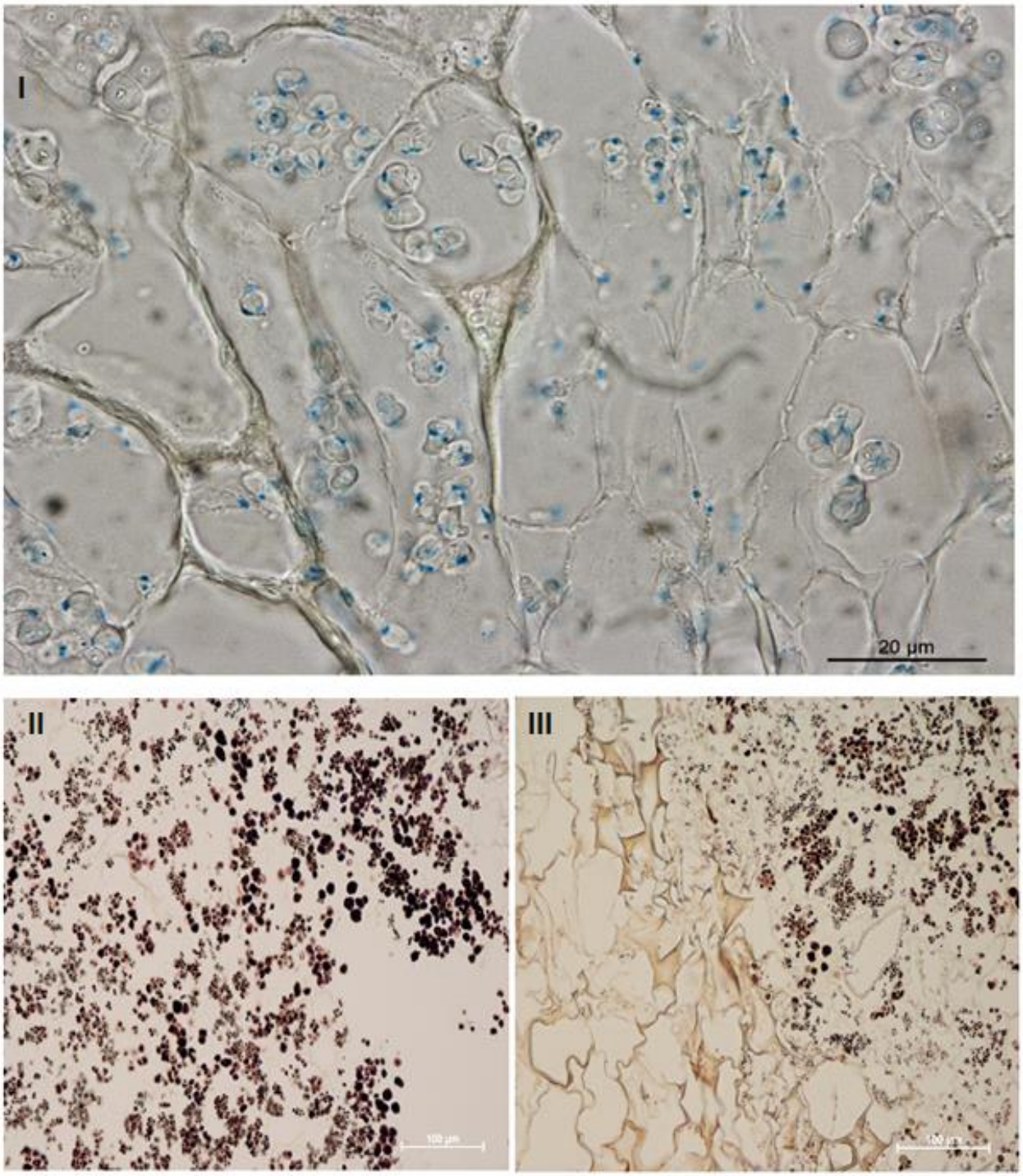


Figure 24. Microscopy of Fe localisation and starch granules deposits in callus tissue grown on sufficient Fe medium. I: Prussian blue precipitates located within small spherical and actively dividing cells; II: starch granules distribution; III: starch granules localisation in central regions of callus tissue.

Iron functions as a cofactor for several cellular processes and therefore it is required in various sites within an organ or a cell. Appropriate Fe distribution in the different organelles is crucial for maintaining cell function and integrity. There are no previous studies on Fe localisation in calli exposed to varying Fe supplies. However, relevant studies on plant organs have been reported. Roschzttardtz et al. (2013) have reported on Fe distribution in plant roots, leaves and flowers using Perls/DAB staining. Leaves of plants grown without iron supplementation revealed very minimal staining of Fe in mesophyll cells but intense staining in the region of the vascular system (Roschzttardtz et al., 2013). Plants grown on standard Fe supply conditions showed Fe staining in the leaf chloroplasts, the vasculature and nearby parenchyma cells. Leaves of plants exposed to an elevated iron concentration displayed intense staining in the plastids and excess Fe produced abundant staining of Fe in the parenchyma cells of the vasculature region. Supplies of Fe to the plants resulted in increased levels of Fe in the mesophyll cells and the appearance of Fe-rich granules in plastids. The vacuoles and plastids are important organelles for the intracellular compartmentalisdisation of iron (Briat et al., 2007). The alteration of iron distribution in the vacuole and the chloroplast of *Arabidopsis thaliana* was observed to severely affect plant growth (Jeong et al., 2008). Pea (*Pisum sativum*) embryo was found to have measurable amounts of iron in the nucleus (Roschzttardtz et al., 2011a). According to the authors, the presence of Fe in plant nuclei might reflect a plant-specific function of iron and not associated with a dysfunction. They hypothesised that in plants, iron may be involved in ribosomal RNA metabolism since the nucleolus functions primarily in the biosynthesis of rRNA. Synthesis of rRNA may be achieved by Fe either advancing or stabilising the secondary structure or by catalysing maturation of the different rRNA subunits (Roschzttardtz et al., 2011a).

Callus tissue proliferates as highly disorganised mass of cells with irregular shapes. Callus cultures are suggested to induce unorganised growth and to produce instability in plant tissue at a considerably high rate (Bairu et al., 2011; Vázquez, 2001). In the current study, a heterogeneous mixture of cells of different shapes, sizes and stages of development were identified in potato callus tissue. Light microscopic observations of cells stained with I_2-KI established presence of starch granules in potato callus. Numerous starch granules and actively dividing cells were detected in calli grown on medium with sufficient Fe supplies (Figure 24) while the reverse (less starch) was observed in calli with limiting Fe supplies. This confirmed a previous study, which indicated that potato callus contain starch granules. The starch content of callus derived from potato tuber tissue has been evaluated by Hagen et

al. (1991) found to be composed of 0.5 to 4% starch on dry matter basis. Hagen et al. (1991) revealed that changes in potato callus starch content paralleled changes reported in tubers.

3.11 Summary

It is necessary to know the visual, morphological and biochemical manifestations or presentations associated with the Fe efficiency trait of interest in order to adequately identify and select for it. Morphological and biochemical attributes distinguished between calli submitted to Fe deficiency and calli optimally supplied with Fe. The results herein show clearly that the development of Fe-deficiency response in potato calli is controlled by the concentration of Fe supplied in the culture medium and the duration in culture. The findings indicate that Fe is essential and/or influences growth, morphology and biochemical processes in potato callus cells. Callus cultures subjected to limiting Fe supplies exhibited the classic visual chlorosis symptoms, with a more severe impact over a long-term culture duration and is particularly strong in calli cultured in the absence of Fe. Fe stress (deficiency and excess) restricted callus growth by significantly reducing fresh weight and area of growth with a pronounced effect over long term. Contrarily sufficient Fe levels had a stimulating effect on growth, fresh weight and area of growth area covered by callus cultures.

Fe shortage had adverse effects of on callus biochemistry (decreased chlorophyll and carotenoid content, reduced antioxidant enzyme activities, increased lipid peroxidation). Exposure of potato calli to Fe deficiency enhanced ferric reduction activity, induced phenolic production and increased H_2O_2 staining intensity. Antioxidant enzyme (CAT, POD APX) activities correlated with strongly with Fe content in medium especially when Fe depletion continued. Based on the Perls staining technique, Fe was noted to be widely distributed in actively growing cells of calli cultured on sufficient Fe medium as compared to Fe-deficient medium. The biochemical responses of calli to Fe deficiency were to a large extent more pronounced in long-term than in short-term cultures. The aforementioned biochemical responses in calli were gradually reversed by increasing Fe supplies in the medium from 0 to 50 µM. The results presented here suggest that the morphological and biochemical parameters assessed can serve as indicators of the Fe nutritional status of calli.

CHAPTER FOUR

Fe deficiency stress induced somaclonal variation relating to differential tolerance to chlorosis in callus cultures

4.0 Introduction

To reduce the agricultural and economic impact of iron deficiency stress, it has become necessary to deploy biotechnological tools to develop plants that are adapted to such stress. Tissue culture has become known as a cost-effective and practical tool for the selection of stress-tolerant variants because millions of cells can be screened within a relatively limited space and time. Development of Fe-efficient plants is suggested to be the best practical means to avert Fe availability problems (Hansen et al., 2006; García-Mina et al., 2013; Vasconcelos and Grusak, 2014). An iron-efficient plant is highly capable of optimum use of Fe for its metabolism. Such a plant can take up a smaller amount of the nutrient compared to a less efficient plant and produce the same yield (García-Mina et al., 2013; Vasconcelos and Grusak, 2014). Evidence for the use of *in vitro* approach in the selection of Fe-efficient cell lines through exposure of callus to Fe deficiency stress conditions can be drawn from studies on sugarcane (Naik et al., 1990). Vasconcelos and Grusak (2014) identified novel sets of Fe-efficient cell lines in soybean and confirmed their tolerance to IDC under laboratorial condition.

In vitro selection is achievable based on the pre-existing variation in explants and/or heterogeneity of callus due to somaclonal variation (Wang and Wang, 2012). Callus cultures are the most convenient for use in *in vitro* selection programmes and have higher somaclonal variation or mutation rates (Bairu et al., 2011; Vázquez, 2001). Using *in vitro* culture, somaclonal variation can be exploited to produce novel clones with desirable agronomic traits that are beneficial for crop improvement (Thieme and Griess, 2005; Larkin and Scowcroft, 1981**).** Ways to improve the recovery of somaclonal variants include: prolonged culturing of callus, *in vitro* selection of tolerance against stresses, regeneration of plants, assay for genetic stability of selected somaclones, and multiplication of the somaclones for developing new cultivars (Brar and Jain, 1998; Jain, 2001).

Tissue culture system allows the use of a selective agent, unique selection procedures and the precise regulation of growth conditions. An effective selection system must take into account the difference between a variant and a mutant, the connection (or lack of it) between cellular and whole-plant phenotypes, cellular manifestation of the phenotype and complexity

of the phenotype (Jain, 2001; Karp, 1995; Widholm, 1989; Berlin and Sasse, 1985; Meredith, 1984). They are normally employed principally for resistance and visual selection of a variety of traits. Meticulous design and application of a suitable selection strategy which support the preferential growth and survival of a desired phenotype is necessary to ensure the isolation of specific variants. Selection for tolerance or resistance employs a selection strategy that basically challenges cells in a culture medium with inhibitory levels of a compound or condition. This approach, also referred to as positive selection, purposely favours the survival of the desired variant trait while the growth of wild type cells is inhibited. To obtain Fe-efficient potato callus lines, the direct selection strategy was employed (see section 1.9.6). This involved the sudden exposure of callus to the selective pressure (Fe-deficiency) that enable the survival of only a proportion of the population that can tolerate and are adapted to such conditions. Research findings suggest that this strategy can be used to prevent the development of epigenetically adapted cells (McHugen and Swartz, 1984; Chandler and Vasil, 1984; Tal, 1994).

Potato can serve as an ideal crop for the enhancement of iron bioavailability. The advantage potato provides is that iron in potato can be more bioavailable than that in cereals and legumes because of the presence of high levels of iron absorption promoter, ascorbic acid and low levels of phytic acid, an inhibitor of iron absorption (King and Slavin, 2013; Frossard et al., 2000; Love et al., 2004; Phillippy et al., 2004; Burlingame et al., 2009; Navarre et al., 2009). Significant genetic diversity in Fe levels in potato suggests that it is possible to increase the iron value of potato (Haynes et al., 2012). It is therefore imperative to select for somaclones with efficient use of iron and enhanced Fe content.

This section describes procedures employed in the exposure of potato calli to varying selective pressures (Fe-deficiency treatments) and selection of somaclonal variants and propagation of the somaclones. The callus culture system was used because the overall objective of the research required the regeneration of plants and plant regeneration is most reproducible from callus cultures (Widholm, 1989; Berlin and Sasse, 1985).

4.1 Methodology

4.1.2 Selection of Fe-efficient callus cells

Preliminary experiments (see Sections 3.5) in which calli were exposed to a wide range of Fe concentrations (0-800 µM) together with information gathered from literature (Legay et al., 2012; Bienfait et al., 1987; Kerkeb and Connolly, 2006; Zuo and Zhang, 2011) served as a basis and guideline for choosing the treatments applied as Fe-deficiency selective pressure (i.e. 0-5 µM). A two-step selection scheme was designed which involved the sudden exposure of calli to Fe-deficiency conditions after prior growth on sufficient Fe (50 µM) medium. Young leaf explants were similarly exposed to media deficient in Fe. A range of Fe-deficiency treatments (0-5 µM) was used as selective pressures. Calli and leaf explants were exposed to iron deficiency treatments to allow for the survival (selection) of only somaclones adapted to the selective pressures applied. The selection process was designed to deliberately favour the survival of the variant of interest.

Primary callus cultures proliferated over an 8-weeks period on CIM (of 50 µM FeNaEDTA) supplemented with 3.22 µM of NAA and 1.78 µM of BA content) were subcultured directly onto a selection medium (SM) of similar composition but with different concentrations of FeNaEDTA. The selection trial with leaf explants involved direct culture on SM. There were nine treatments (i.e. 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 µM) representing different Fe-deficiency selective pressures applied and the control (50 µM). Three pieces of excised calli (approximately 120 ±10 mg) were cultured per Petri dish containing 20-25 ml of SM. Each callus was 3- 4 mm in diameter. Each treatment had 30 biological replicates (in 10 Petri dishes) and each experiment was repeated at least four times. At least 120 biological callus replicates were cultured per treatment. Petri dishes were placed in a temperature controlled room at 22 (± 2) ˚C under constant illumination (24 hr photoperiod).

Fe-deficiency tolerance selection involved the excision of small pieces of growing and green callus (selected cells) from surrounding dead cells. The growing cultures were subcultured on the respective medium at 4-week intervals up to 3-6 months (see Figure 5). The selection procedure was improved by subculturing minute pieces of callus in order to reduce the risk of formation of chimaeras as suggested by Collin and Dix (1990). Since not all pieces of callus might be in direct contact with the medium, growing callus cells were subcultured for extended periods (3-5 months) on the selective agent to eliminate the sensitive cells as Berlin and Sasse (1985), showed that repeated culturing of callus cells on

inhibitory concentrations of the selective compound or condition can eliminate the sensitive cells. By visually inspecting calli cells, Fe-deficient calli that maintained proliferation ability and showed no chlorotic symptoms similar to the control, Fe-sufficient calli, were denoted as chlorosis-tolerant (Fe-efficient) calli. Fe-efficient callus cultures were isolated for plantlet regeneration.

4.2 Plantlet regeneration from potato callus cultures

Potential Fe-efficient callus lines selected after 3-6 months of exposure to Fe-deficiency conditions (0-5 µM) as well as calli sustained on sufficient Fe supplies (50 µM) were cultured on RM composed of half-strength MS supplemented with 6.66 µM BA and 2.89 µM GA_3. Subsequent shoot proliferation, induction of rooting and micropropagation of regenerants are as described in Section 2.5.

4.3 Preliminary optimisation trials and findings

4.3.1 Strategies for selection of Fe-efficient variants

Two schemes were examined in the initial screening for Fe-efficient cell lines. One-step and two-step direct selection strategies were employed involving the sudden exposure of leaf explants and calli respectively to Fe-deficiency conditions**.** It was thought that by directly exposing leaf explants to Fe-deficient conditions, calli induced and capable of thriving may be inherently more resistant to the selective pressure applied compared to calli formed under normal Fe (50 µM) growth conditions before being exposed to low Fe supplies (two-step selection). Callus was initiated both under Fe-deficient (0-5 µM) and sufficient Fe, control (50 µM), conditions within 3-4 weeks of culture. Variations in pigmentation and growth were identified by visually inspecting the cultured cells. The colour of the calli produced (see Figures 25 and 26) ranged from light yellow, light green to green on 0-5 µM Fe CIM and green to very green on medium with 50 µM Fe concentration (control). As indicated earlier (Section 3.5.2), changes in pigmentation occurred more rapidly in primary induced calli compared to subcultured calli. Primary calli induced from leaf explants were found to be more susceptible (quicker response: symptoms in 3-4 weeks) to Fe deficiency-induced chlorosis (yellowing) compared to calli exposed to Fe deficiency after growth on 50 µM Fe medium (symptoms after 4 weeks). As stated earlier (Section 3.5.2), leaves seem to provide a

larger surface for interaction with iron than callus cultures. Leaf explants are possibly more responsive to iron supply than compact calli which might have developed some adaptive features or had Fe stored following the prior exposure to sufficient (50 µM) amounts of Fe. It is assumed that the mode or pathway for the uptake and utilisation of iron may be different in leaf and callus cells. Subcultured calli proliferated at a faster rate relative to induced (primary) calli. The increase in growth and the size of calli produced were observed to be affected by the age (young) and/or size of leaf cultured. The two-step direct selection approach was preferred and employed for further Fe-efficiency selection studies based on the lower proliferative capacity of leaf explants to generate callus compared to growth of subcultured calli and the difficulties in obtaining copious amounts of evenly-sized leaf explants of similar age. The thorough selection regime carried out is detailed in Section 3.5.2.

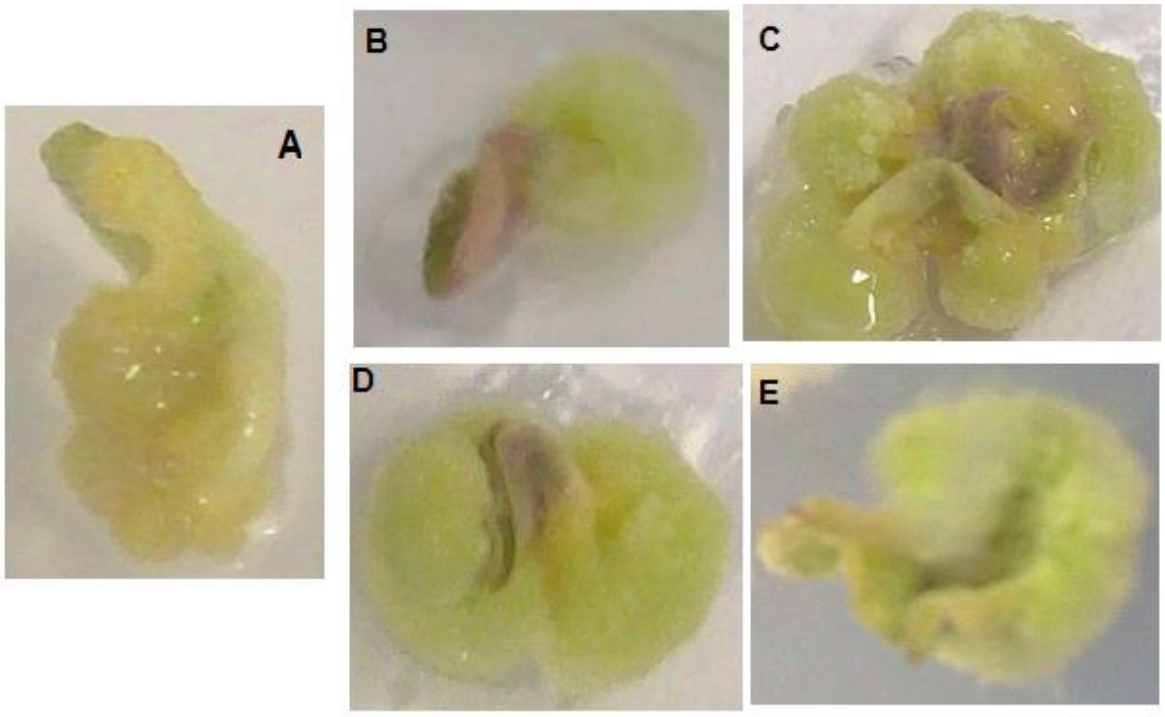


Figure 25. Callus initiation from potato leaf explants exposed to Fe-deficient (A: 0 µM; B: 0.05 µM; C:0.5 µM; D:5 µM) and sufficient Fe, control (E: 50 µM) conditions for 4 weeks.

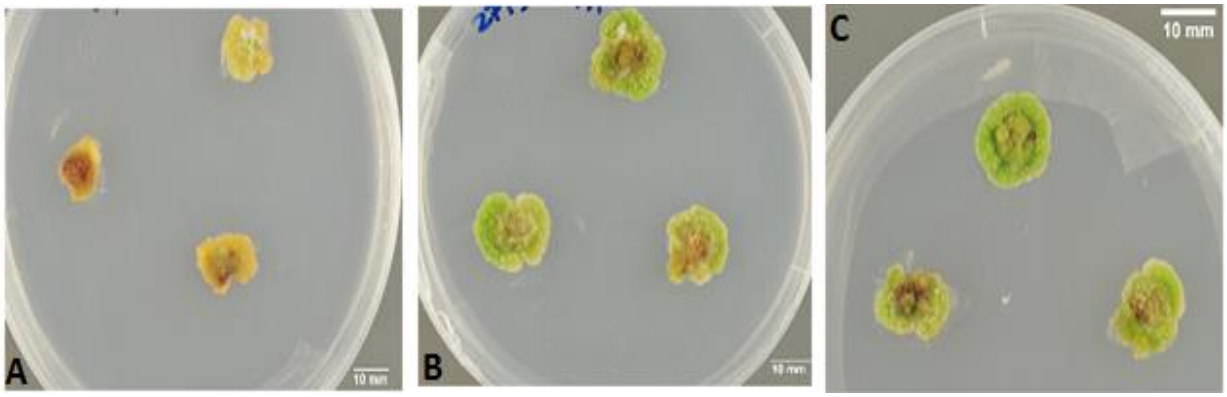


Figure 26. Callus growth on Fe-deprived (A: 0 µM), Fe deficient (B: 5 µM) and sufficient (C: control, 50 µM) Fe medium. Calli first proliferated on control medium (supplemented with 50 µM Fe) for 8 weeks before they were subcultured on the Fe-deficient media for 6 weeks.

4.3.2 Optimisation of shoot regeneration culture conditions

The effect of different PGR combinations on shoot regeneration efficiency was studied in order to determine an efficient protocol for optimal plant regeneration. The ability of plant cells to regenerate due to their high developmental plasticity is exploited and readily achieved in this study by proper hormonal regulations. Callus cultures were tested for their potential to regenerate under *in vitro* conditions and an effective protocol for plant regeneration was developed. Plantlets regenerated from vigorously growing calli with pronounced morphogenic potential. There were some slow growing calli which did not present/show any morphogenic potential and as such, did not produce plantlets. For most calli that regenerated into plantlets, globular and heart shaped structures consistent with the involvement of somatic embryogenesis were first observed followed by shoot initiation (see Figure 27). Although some calli developed globular structures, they did not produce shoots. It seems worthwhile in future studies to investigate in more detail the histological ontogeny of plantlet regeneration of 'Iwa' potato using the present protocol.

The regeneration potential of calli varied widely among the media compositions used. The first visible shoot buds were observed within 4-7 weeks on globular and heart shaped structures developed on 83.3% of calli cultured on medium supplemented with 6.66 µM BA and 2.89 µM GA_3 (B5G5). A response time of 12 weeks was recorded for the development of shoot buds in about 60% of the media formulations that gave rise to regenerants but at a

faster time in B5G7 and B5G8 compared to B5 medium (Table 8). The results suggest that, while maintaining the same amount of BA, (6.66 µM) even small changes (increase or decrease) in GA_3 concentrations influenced the regeneration frequency and shoot formation considerably. With BA as the sole PGR, the regeneration frequency ranged from 20 to 40% (Table 8). The regeneration frequency of calli cultured on B5 (6.66 µM BA) was 18% more with a quicker response time (3 weeks early) than that of calli on B4 (3.55 µM BA) medium. Even though more shoots (3.99) were regenerated in B5 than in B4 (3.0), the difference was not significant.

The results revealed that the RM, B5G5, was the most effective (optimal) medium for efficient shoot regeneration producing the highest yield of regenerated plants (6.33) per callus and with a significantly higher regeneration frequency (83.3%) compared to calli in other culture media (Table 8). In this medium, the percentage of calli regenerated into shoots was significantly more (2-3 times) than in RM containing only BA. Calli should be at least 4-5 mm in diameter since smaller ones did not survive and the few that developed globular structures lost their regenerative potential even when cultivated on the optimal medium, B5G5. The presence of BA in the RM was essential for shoot induction from callus since culture media supplemented with only GA_3 or NAA did not produce any shoots (Table 8). Likewise, media formulations with combinations of either NAA and BA (NBR1-5) or all three PGRs (NBG1-7) did not generate shoots. Calli cultured on NBG1-7 (varying combinations of NAA, BA and GA_3) media did not develop any visible heart shaped structures nor shoot buds even after 15-20 weeks. Calli in these media turned dark brown, showed signs of senescence and became necrotic. Similar results were obtained for calli cultured on media containing only NAA (N2-5) or GA_3 (G1-7). The results confirmed that plant regeneration competence is strongly influenced by the types, concentrations and combinations of PGR as reported by Kumar et al. (2014). When shoots had grown 1.5-3cm tall on regeneration medium (Figure 27), they were transferred to a PGR-free medium for shoot elongation and rooting. Plants developed on the various RM were normal in growth and resembled the stock (parental) plants in terms phenotypic characteristics. However, plants regenerated on B5G7 medium presented gross morphological aberrations (including no or tiny leafless shoots, profuse bushy stems).

In conclusion, PGRs were found to play an important role in plantlet regeneration from callus cultures. The results indicate that plant regeneration from callus require the suitable concentrations and combinations of PGRs in the culture media and appears to be dependent on the morphogenic potential of the callus in agreement with reports by Collin and

Dix (1990). Based on the response obtained from the preliminary experiment, selected Fe-efficient calli selected after 3-6 months of exposure to Fe-deficiency conditions were cultured on B5G5 media.

Table 8. Influence of plant growth regulators on shoot regeneration from potato callus cultures.

culture medium	concentrations of PGRs (µM)			shoot regeneration frequency (%)	response time (weeks)	no. of shoots regenearated per callus*
	NAA	BA	GA_3			
½ MS	0	0	0	0	0	0
B1	0	0.89	0	0	0	0
B2	0	1.78	0	0	0	0
B3	0	2.66	0	0	0	0
B4	0	3.55	0	25.00 (±0.50)^{c}	15–17	3.00 (±1.00)^{b}
B5	0	6.66	0	41.67 (±14.23)^{b}	12–14	3.67 (±1.15)^{b}
B6	0	8.88	0	0	0	0
G1	0	0	0.58	0	0	0
G2	0	0	1.16	0	0	0
G3	0	0	1.73	0	0	0
G4	0	0	2.31	0	0	0
G5	0	0	2.89	0	0	0
G6	0	0	4.34	0	0	0
N2	2.15	0	0	0	0	0
N3	3.22	0	0	0	0	0
N5	5.37	0	0	0	0	0
B5G5	0	6.66	2.89	83.33 (±15.43)^{a}	4–7	6.33 (±1.53)^{a}
B5G6	0	6.66	4.34	8.33 (±14.5)^{d}	15–17	1(±1.73)^{c}
B5G7	0	6.66	5.78	41.67 (±14.43)^{b}	10–12	4 (±1.00)^{b}
B5G8	0	6.66	7.23	33.33 (±14.43)^{b}	11–13	3.33 (±0.58)^{b}
NBR1	3.22	1.78	0	0	0	0
NBR2	4.30	0.89	0	0	0	0
NBR3	8.06	1.78	0	0	0	0
NBR4	13.43	8.88	0	0	0	0
NBR5	16.11	4.44	0	0	0	0
NBG1	3.22	1.78	0.58	0	0	0
NBG2	3.22	1.78	1.16	0	0	0
NBG3	3.22	1.78	1.73	0	0	0
NBG4	3.22	1.78	2.31	0	0	0
NBG5	3.22	1.78	2.89	0	0	0
NBG6	3.22	1.78	4.34	0	0	0
NBG7	3.22	1.78	5.78	0	0	0

Regeneration frequency was estimated as the ratio of the number of calli that regenerated shoots to the total number of calli cultured. Values (mean± SD) followed by the same superscript letters are not statistically significant at $p<0.05$ according to ANOVA and Duncan post-hoc test. Values (mean± SD) are calculated after three subcultures in regeneration medium.

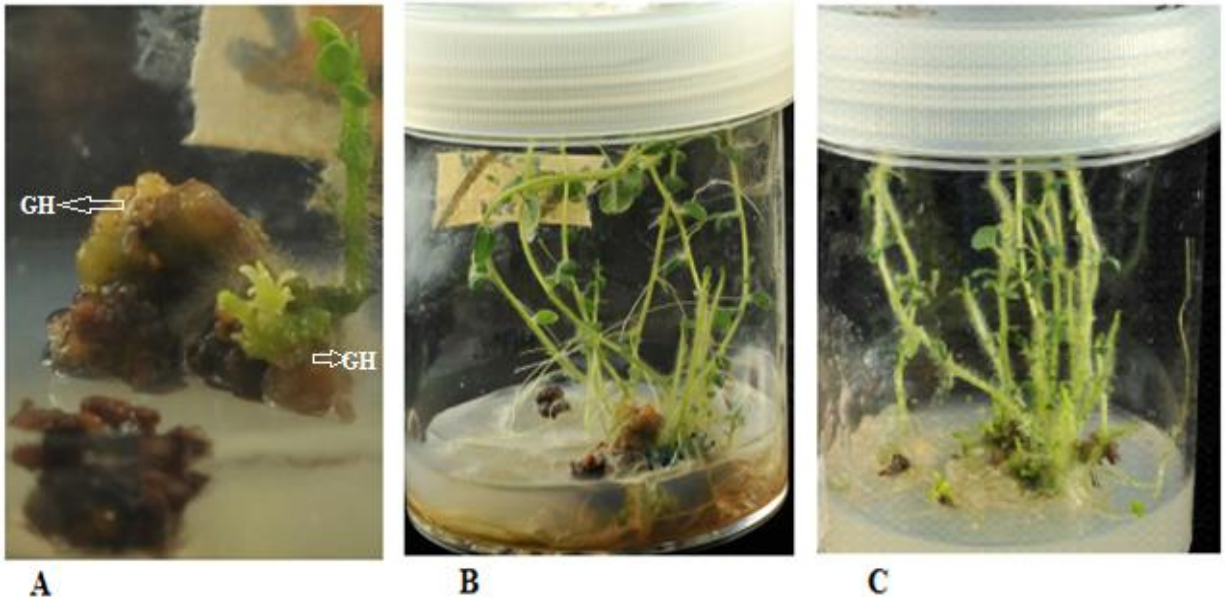


Figure 27. Development of globular and heart-shaped structures (GH) and shoot buds from callus (A). Regeneration of plantlets on B5G5 (B) and B5 (C) regeneration media.

4.4 Main results and discussions

4.4.1 Fe-efficient callus cells exhibit IDC tolerance

Visual screening has been used for the *in vitro* selection of sensitive and tolerant cultivars (Tangolar et al., 2008; Lombardi et al., 2003; Svab and Maliga, 1986). In the current work, the initial visual selection process enabled the quick screening and identification of the desired phenotype (tolerance to Fe-deficiency) over the sensitive callus cells on Petri dishes based on differences in pigmentation and cell survival under Fe-deficiency selective pressure. The results (see Figure 28) show that the green pigmentation in calli decreased gradually as iron supplies were lowered. After the first subculture (within 2 months), 60-80% of calli growing on medium without Fe (0 µM) or of low Fe content (0.001-5 µM) appeared chlorotic (light yellow) and bleached but some calli survived and increased in growth area even after 3 months of culture (similar to findings in Section 3.6.1). Selection for IDC-tolerant calli was based on the direct evidence of cell survival under Fe-deficiency stress conditions (see Figure 28) over a period of 3-5 subcultures (3-6 months). Most calli exposed to Fe-deficient conditions (0-5 µM) showed a high degree of chlorosis over time (especially after 2nd subculture) and experienced a decrease in compactness especially at 0-0.5 µM Fe

concentration. Such calli also showed a depression in growth and some were necrotic or senescence (Figure 28). Sections of a small fraction of calli cultured at low Fe levels (0.005, 0.01, 0.5, 1, 5 µM) maintained the green pigmentation ("green islands") even after the 2nd to 4th subcultures. They were comparable to calli grown at sufficient Fe concentration (Figure 28). These were considered as potential somaclonal variants and were selected for further studies on plant regeneration.

The recovery of Fe-efficient somaclonal variants was improved through prolonged culturing of callus, multiplication of the variant lines and regeneration of plants for developing new cultivars as suggested by Jain (2001). Since not all pieces of calli might be in direct contact with the selective medium, extended culturing of growing callus cells on the selective agent can eliminate sensitive cells as suggested by Berlin and Sasse (1985). The tolerant cells "green islands" were excised from the bleached/chlorotic sections of calli and then regenerated into potato plants for validation of Fe deficiency tolerance and characterisation. A total of about 30 Fe-efficient callus cell lines were selected from the over 120 biological callus replicates cultured per selective pressure applied. About 10% of these lines did not regenerate into plantlets in initial studies in which plant regeneration from Fe-efficient calli were tested on regeneration medium supplemented with low Fe-supplies (0-5 µM). Approximately 56% of the selected Fe-efficient callus cell lines however, produced regenerants on regeneration medium containing 50 µM Fe (see Section 4.4.2).

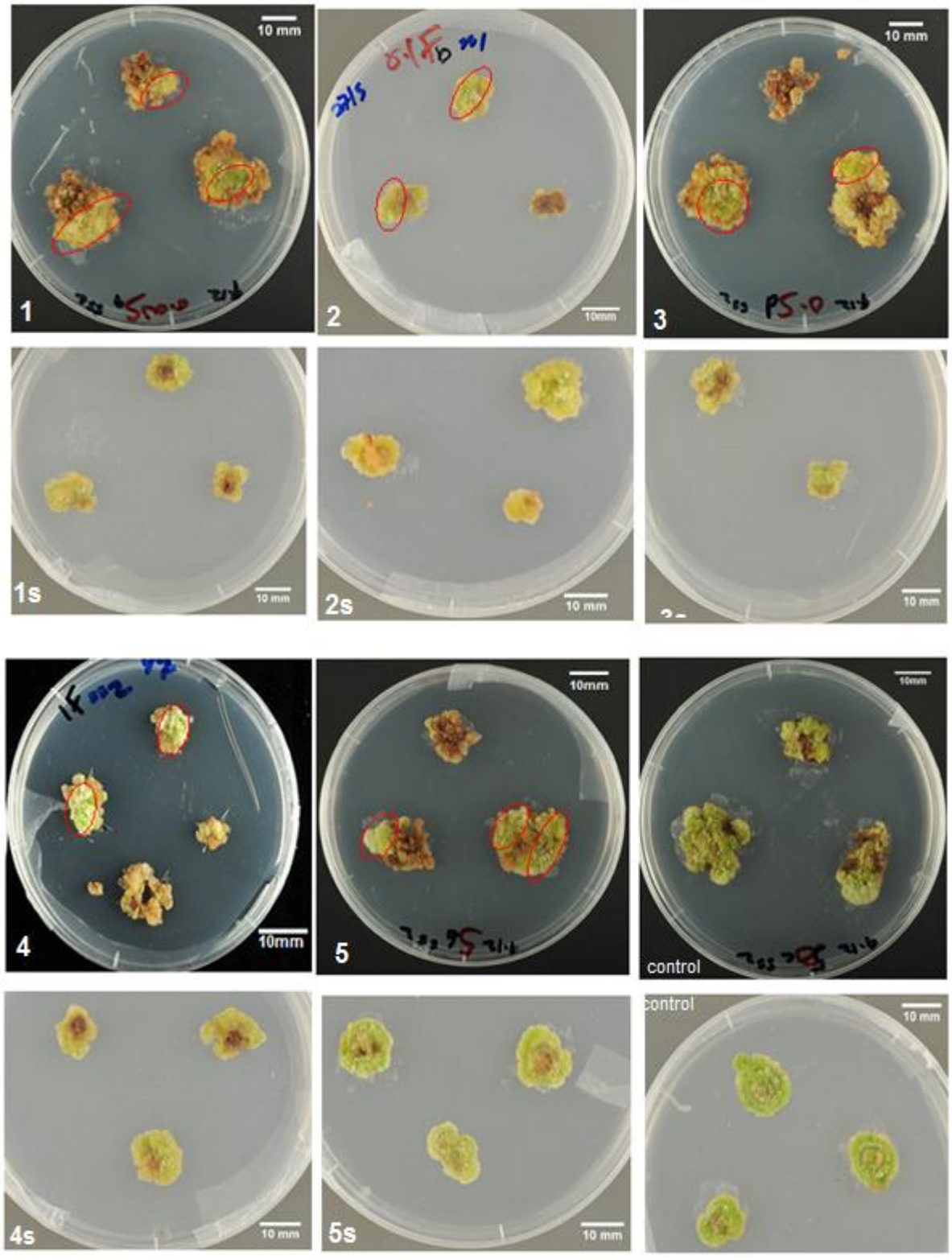


Figure 28. Selection for Fe-efficiency in potato callus cultures under Fe deficiency selected pressure. Isolated Fe-efficient somaclonal variants (circled in red) were subcultured on respective Fe-deficient medium. Calli cells ("green islands") were selected and subcultured on corresponding Fe-deficient CIM for 4 weeks to get rid of chimeras/mixed chlorosis sensitive cells to obtain Fe-efficient callus lines.

The selection of Fe-efficient lines of crop plants using the callus culture technique was attempted in sugarcane plants by indirectly inducing Fe deficiency condition with incorporation of $CaCO_3$ in growth medium (Naik et al., 1990). Although there are reports on *in vitro* screening and/or selection for Fe-efficiency trait in other crops (details reviewed in section 1.7.2) reports on the selection of Fe-efficient lines in potato calli have not been found indicating that the Fe-efficient variants generated in the present study are novel materials. *In vitro* techniques have been used as a fast method for the selection of various cultivars with a higher tolerance to iron deficiency chlorosis (Mahdavikia and Mahna, 2012). *In vitro* culture of calli under aseptic conditions enables the specific manipulation of plant growth and development through a rigorous regulation of environmental and nutritional parameters. These factors are difficult to control under natural conditions and their regulation *in vitro* allows for the rapid isolation of somaclones with desirable traits (Jain, 2001). The best strategy towards *in vitro* selection is to manipulate somaclones with an appropriate selection pressure (Lestari, 2006; Pérez-Clemente and Gómez-Cadenas, 2012; Rai et al., 2011).

Fe deficiency in soils is associated with multiple soil and /or biochemical stresses therefore, the *in vitro* selection regime applied for the selection of Fe-efficient potato callus lines in the present work has been uniquely designed to create direct and specific Fe-deficiency stress levels as selective pressures. A suitable selection agent enables the preferential survival and/or growth of variant cells and plants with desirable phenotypes (Pérez-Clemente and Gómez-Cadenas, 2012; Rai et al., 2011). The Fe-deficiency selective pressures applied increased the possibility of discovering clones with desirable traits by eliminating unwanted (sensitive) cells. Creating or establishing the actual Fe-deficiency status (low concentrations of Fe in medium) and not the causative factors (carbonates, high pH, etc.) was thought to be most effective way of generating chlorosis resistant plant capable of thriving in soils deficient in iron. In some studies, nutrient medium containing $NaHCO_3$, $KHCO_3$ or $CaCO_3$ have been used induce Fe-deficiency since carbonates bind to Fe and limits its availability as occurs in calcareous soils (Naik et al., 1990; Dolcet-Sanjua et al., 1992; Kabir et al., 2015; Palombi et al., 2007).

The results of this study demonstrate that it is possible to generate novel IDC- tolerant somaclonal variants that may be useful for crop improvement. SV in the calli formed from leaf explants of a common donor parental clone occurred spontaneously as a result of *in vitro* tissue culture cycle as proposed by Larkin and Scowcroft (1981). Aside pre-existing variation in explants, SV causes phenotypic diversity of cells *in vitro* which is of much higher frequency than the natural variation that occurs in living systems (Phillips et al., 1994). Using

a mathematical model to describe the connection between SV and culture period, Coˆte et al. (2001) proposed that variation increases as the culture ages as was found in this present study. The probable sources of SV observed in the callus cultures include the plant genotype, culture medium, growth regulators and *in vitro* culture period. The Fe-efficient variants selected were either due to the expression of an existing variation in the source plant or to mechanisms that result in genetic variation as indicated by Larkin and Scowcroft (1981). The tissue culture medium or system can be considered as a mutagenic treatment that initiates a mutation process and can generate genetic variability (Bairu et al., 2011). The *in vitro* culture conditions designed for this study exerted stress on callus cells and this can activate mutagenic processes as suggested by earlier reports. When cells experience trauma or stress, they may reset genome expression in a manner contrary to the sequence that takes place under natural conditions (Phillips et al., 1994). Such reprogramming can give rise to somaclones with altered expressions in plants arising from various genetic modifications and activation of transposable elements (Pérez-Clemente and Gómez-Cadenas, 2012; Phillips et al., 1990; Kumar and Marthur, 2004; Kaeppler, et al., 2000; Rao et al., 1992).

The combinations of PGRs incorporated into the CIM may have contributed or be the source of some of the variations observed in the callus cultures but since the amounts of PGRs used was optimised and the same across all treatments, the influence on SV may have been negligible. Plant growth regulators used in culture media may cause an increase in somaclonal variation resulting from DNA methylation (Bairu et al., 2011). High concentrations of N_6-benzylaminopurine (BA), 15 mg/l and 30 mg/l, increased genetic variability in banana (Gime´nez et al., 2001) and rice cultures (Oono, 1985), respectively. BA was implicated in the increase in chromosome number in a somaclonal variant (Bairu et al., 2011). The role of cytokinins and PGRs in general in somaclonal variation however, is not clear and results are inconclusive (Bairu et al., 2011).

4.4.2 Fe-efficient callus-derived plant regenerants

Traits selected at the cellular level may be lost during plant regeneration and/or at various stages of plant development. In the current study, plants were regenerated from callus cultures to investigate whether the Fe-efficient trait selected in calli will persist in plants. To facilitate the quick recovery of plants, the variant colonies tolerant to Fe-deficiency conditions were transferred immediately to regeneration medium in order to minimise the chance for other unintended random somaclonal changes arising during long term cell culture. Shoot bud development was detected 7 to 14 weeks after culturing selected calli on RM (Figure 29). Bud regeneration appeared in only those calli which showed good cell growth. Selected Fe-efficient calli showing the best bud regeneration from each set of lines (A-E) were chosen for further subcultures. Initially, the number shoot buds produced per calli was low for some of the Fe-efficient callus lines (e.g. calli selected on 0.1 and 1 µM Fe medium). Furthermore, buds were totally embedded in the callus, making it difficult to dissociate them entirely from the callus tissue. The calli were, therefore subcultured either in full or in part in half-strength PGR-free MS medium. Over the course of three to four successive subcultures, shoots were fully differentiated, with roots developed. A total of 17 calli representing excised “green-islands” selected to be Fe-efficent callus cell lines regenerated into plants. Of these, 5 gave rise to defective plantlets showing gross phenotypic modifications (leafless and fluffy stems, tiny numerous/bushy stems) and were discarded.

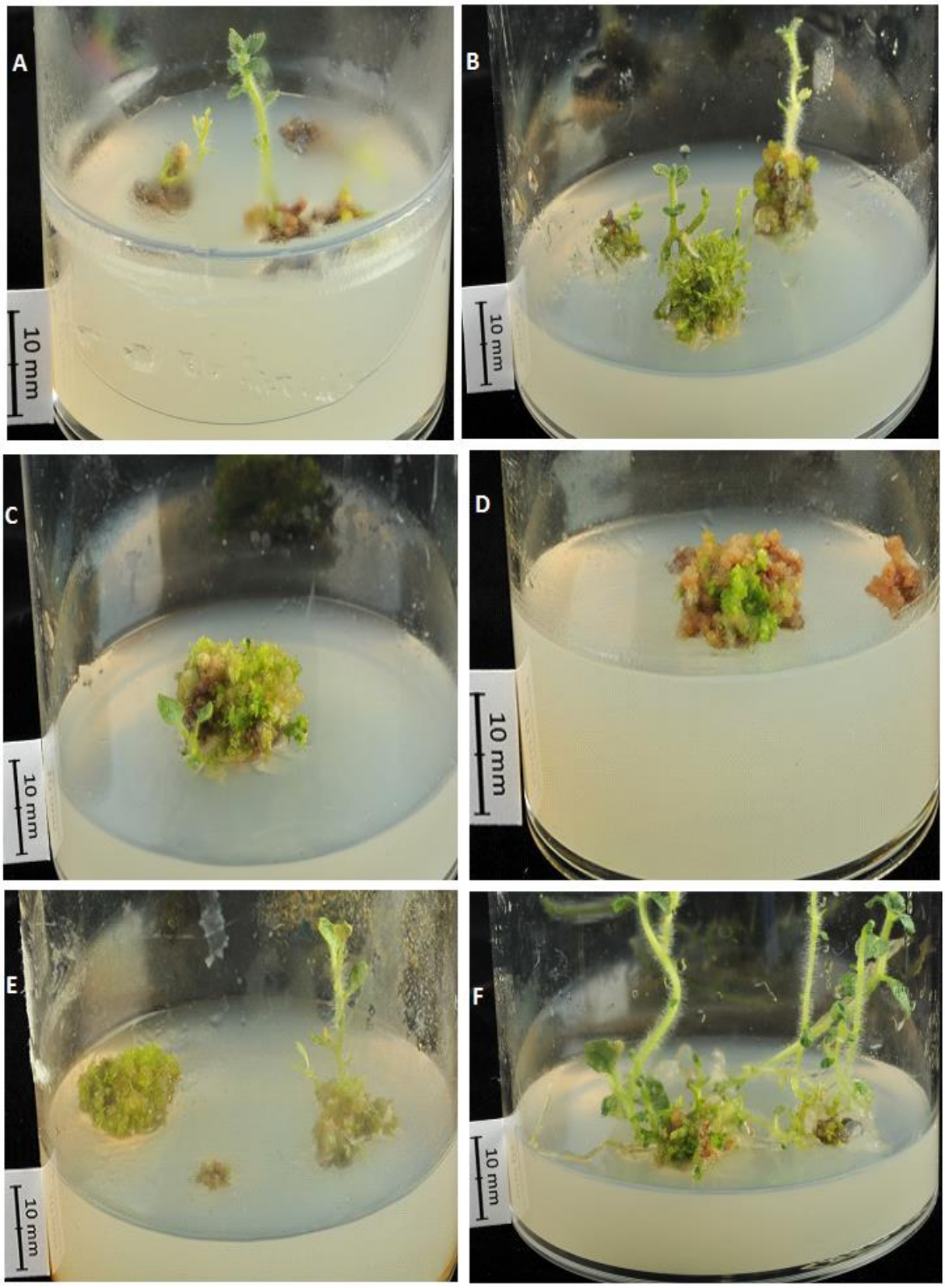


Figure 29. Shoot development from selected Fe-efficient callus lines (A-E) and calli grown on control medium (F). Calli were cultured on regeneration media and shoot buds generated after 1-2 subcultures.

In instances where plant regeneration of resistant cell lines is necessary, selection of variant cells from callus cultures is highly recommended (Berlin and Sasse, 1985). In the present study, plants were successfully regenerated from selected Fe-efficient calli. Such plants can serve as novel materials for the delineation of mechanisms associated with tolerance to Fe deficiency in plants and/or can be useful for cultivation on soils low in Fe nutrition. According to Lestari (2006) tolerance to specific selection pressure at the callus level is generally positively correlated with the tolerance at the plant level. Resistance mechanisms observed in salt-resistant variants were comparable to those known to operate in intact plants (Meredith 1984**)**. Reprogramming of plant regenerants upon exposure to traumatic changes in a tissue culture environment, produce genetic and epigenetic variations (Jain, 2000, 2001; Meredith, 1984).

Plants may not be regenerated from older cultures (Wang and Wang, 2012) as was detected in the current study in most calli cultured for 6 months and over under Fe-deficiency stress conditions. Additionally, calli with intense SV can lose vigour and regenerability over a certain threshold (Kaeppler et al., 2000; Wang et al., 2011). Regeneration potential was noted to be high in potato cells that were selected over a short-term period relative to selection from the long term cultures (Wersuhn et al., 1994). Following the assessment of regeneration frequency in Ni tolerant callus lines, Rout et al. (1998) found that Ni-tolerant calli did not lose their regenerative capacity in spite of being exposed to prolonged culture periods.

Previous studies have reported on generation of novel crops regenerated from callus cultures with resistance to mineral stresses (see Table 3). Seedlings obtained from shoots that regenerated from selected Fe-efficient callus lines were chlorosis tolerant and thrived on the Fe-deficiency stress conditions (Naik et al., 1990). Two somaclonal variants recovered from shoots regenerated from quince leaves exposed to limited Fe supplies were found to be Fe-efficient (Dolcet-Sanjua et al., 1992). Palombi et al. (2007) employed *in vitro* regeneration to obtain and establish somaclones highly tolerant to calcareous soils. Plants regenerated from aluminium-tolerant carrot calli were found to exhibit resistance to aluminium (Ojima and Ohira, 1983). Rice plants regenerated from salt-resistant callus expressed some degree of resistance (Yano et al., 1982). Nabors et al. (1980) reported sexual transmission of salt resistance in regenerated *N. tabacum* plants. Somaclones with desirable traits that remained stable and were inherited by progenies have been generated in potato and indica rice (Rietveld et al., 1993; Roy and Mandal, 2005).

4.4.3 Micropropagation and bulking up of regenerants

Regenerants were propagated *in vitro* in order to multiply plants to obtain abundant material for further tests and assessments. Also, regenerants were micropropagated to maintain uniform plants in an axenic culture for the study of somaclonal variation. About three monthly subcultures were required before simple leaves and uniform multiplication was consistently achieved. About 5-6 monthly subcultures were done to multiply the regenerants to obtain ample plant material for post regeneration screening (details in Chapter 5) to test the lines under Fe-deficiency conditions for confirmation of their Fe-efficiency status. Clusters of leaves formed at the shoot tips of some regenerants (see Figure 30) reduced the number of usable nodes for subsequent micropropagation. Calli which did not produce sufficient shoot bud elongation and proliferation to the same extent within three sub-cultures of the micropropagation stage were excluded from the subsequent experiment.

High quality shoot buds or embryogenic tissues are proliferated and maintained by frequent subcultures to sustain morphogenesis. Potato is amenable to micropropagation and regeneration of plants from cultured explants (Karp et al., 1989; Ahloowalia, 1982). *In vitro* propagation and microtuberisation of potato makes it possible for large amounts of materials to be assessed regardless of the crop season (Gopal and Minocha, 1998). In the present study, individual and unique phenotypic expressions in regenerants were stable after numerous passages of micropropagation of apical nodal explants (see Figure 31). Hence mass propagation through tissue culture for true-to-type production of regenerated potato plantlets is feasible.

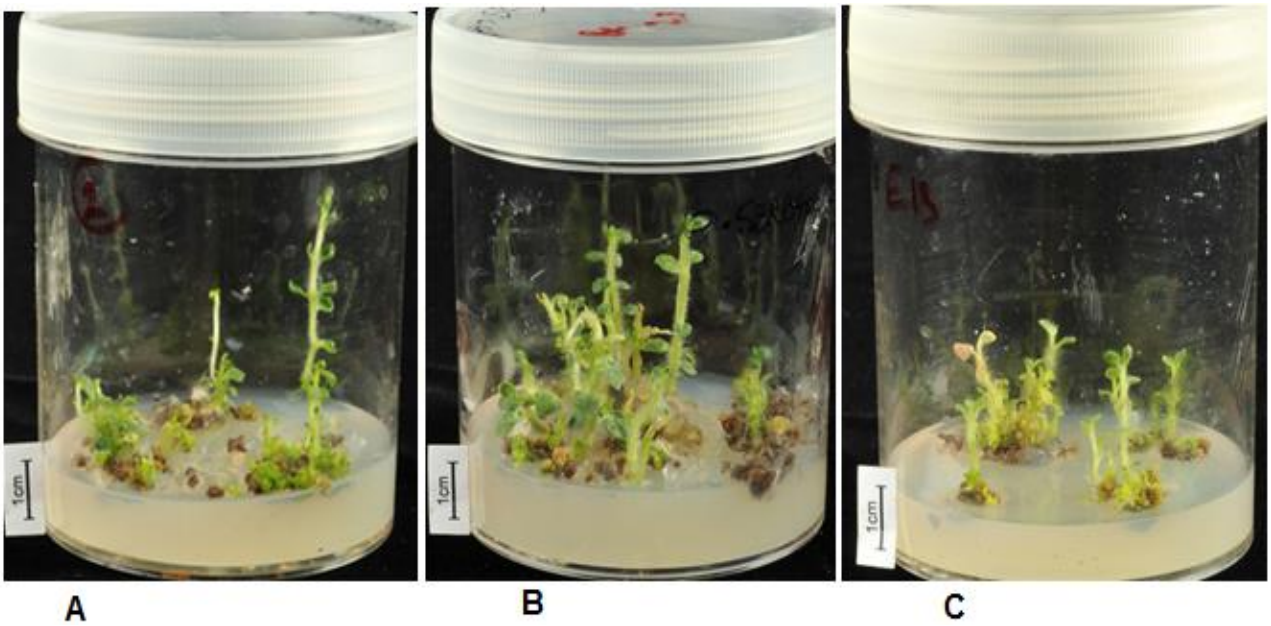


Figure 30. Shoot elongation in regenerants derived from Fe-efficient calli selected from 0.1 (A), 0.5 (B) and 5 µM Fe medium (C) after 11 weeks (2 subcultures) on half-strength MS medium without PGRs. Calli-inducing shoot buds were transferred from regeneration medium unto PGR-free half-strength MS for further development.

4.4.5 Classification of plant lines

The shoots produced per callus were categorised into different plant lines based on growth (fast, intermediate and slow growing) and /or their emergences from different regions of the callus material. Regenerants were also classified into different plant lines depending on which quadrant of the calli a shoot regenerated. With the A plant lines however, one viable vigorously growing and stable plantlet was obtained per callus and was classified by a unique cell line reference (Figure 31). Stable lines were then selected for establishment through micropropagation based on their growth capability and multiplication rates.

The regenerants were grouped according to the source of the chlorosis tolerant callus lines from which they were derived. Plant lines were classified into five groups (A-E) based on the source of the callus from which they were regenerated. Regenerants developed from chlorosis tolerant calli selected on 0.005 µM Fe medium were designated as the A plant lines (see Figure 31). Similarly, regenerants originating from Fe-efficient calli selected on 0.5, 0.1, 1 and 5 µM Fe media were termed B, C, D and E plant lines respectively. The control plants comprised of regenerants from calli continuously grown on medium with sufficient amounts (50 µM) of Fe (referred to as line 50) and the parental stock plants (termed SK). In all, 45

plant lines were established after micropropagation and bulking up of regenerants. Ten of these lines however, were not included in the selection for clorosis resistance because they constituted aberrant phenotypes, failed to establish and/or were of low vigour. The remaining 35 plant lines were tested for resistance to Fe-deficiency chlorosis to confirm their Fe-efficiency nature (post regeneration screening) as detailed in Chapter 5.

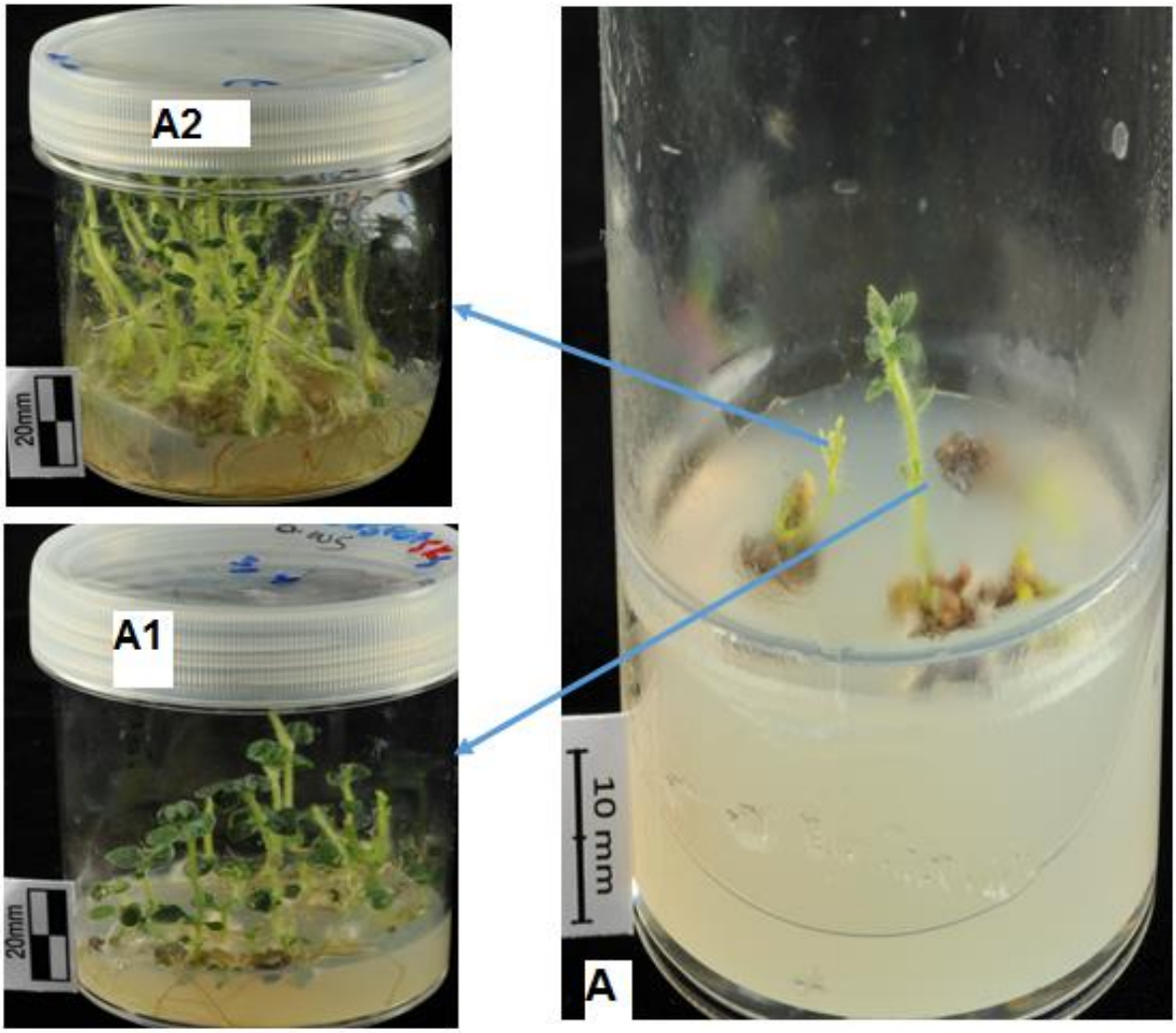


Figure 31. Establishment of A plant lines (A1 and A2) derived from Fe-efficient callus line selected from 0.005 µM Fe medium (A).

4.4.4 Somaclonal variation and phenotypic characteristics of regenerants (plant lines)

Regenerated potato plants in whole, resembled the 'Iwa' parent biotype (referred to herein as SK) but some morphological diversities were observed. The 'Iwa' cultivar is upright and grows vigorous with moderately tall stems and one major leaf per node. The growth habit of all the regenerants was erect as the parental stock plant (SK). Stems ranged from nearly hairless to densely hairy and a single leaf emerged from each node on all plants. The regenerants have young leaves with waxy and hairy surface and matured leaves with smooth and hairless surface.

Somaclonal variation was observed in the plantlets regenerated from selected Fe-efficient callus cultures (Figure 32). The regenerated shoots showed phenotypic and morphological variations which could be very useful for agronomic purposes. Phenotypic differences leading to considerable morphologic variations were detected among regenerants originating from the same callus. Phenotypic diversity in growth, vigour and leaf characteristics was observed. The regenerants expressed a wide range of phenotypic variability including tall and/or bushy plants, small plants with short internodes, plants with thin, slender or thick stems, plants of small, broad and/or pubescent leaves and plants with numerous or fewer leaves. Most plantlets developed simple leaves spirally arranged on the stems, but a few had compound leaves.

Phenotypic variety of a population is due to inherent natural variation either directed by the organism or induced by stress (Rapp and Wendel, 2005). The *in vitro* PTC environment of is not only the extrinsic causative factor of phenotypic diversity but also the conditions for the *in vitro* selection (Wang and Wang, 2012). SV in regenerated plants is based on type of explants used, stage of callus and plant regeneration process (Gao et al., 2010; Wang et al., 2012). Shepard et al. (1980) reported extensive phenotypic variation in plants regenerated from cultured protoplasts of *Solanum tuberosum*. Extensive phenotypic or morphological variation in potato plants regenerated from protoplasts, cells, tissues and tuber discs have been observed (Shepard et al., 1980; Bajaj, 1986). Clones with major diversity in growth pattern, maturation date, tuber size, skin colour and shape and disease response were discovered in Russet Burbank (Shepard et al., 1980; Karp et al., 1989). Somaclonal variation can lead to alteration of plant characters such as plant height, yield, number of flowers per plant, grain quality, resistance to salt, cold, drought, diseases, insect and pests (Jain et al., 1998; Patnaik et al., 1999; Wilson et al., 2009). Rietvald et al. (1993) identified potato somaclones with variations desirable for tuber number and shape, yield and vigour. These variants were stable for over two successive rounds of asexual propagations**.** Slow growing

dwarf dihaploid potato mutants with characteristics of short internodes, dark green leaves, long-day adaptation and compact and ball-shaped appearance in tetraploid were identified by Valkonen et al. (1999). Changes in plant yield, plant height and tiller number of plants regenerated from suspension cultures of *Palmarosa grass, Cymbopogon martinii* were reported by Patnaik et al. (1999). Tuber number, size, shape, eye depth, starch content, starch yield and tuber appearance varied in about 13,000 somaclones obtained from *in vitro* callus cultures of 17 cultivars (Thieme and Griess, 2005). The diverse characteristics observed in the somaclones compared with that of the controls appeared to be dependent on donor genotype and trait. The occurrence of proportions of desirable aberrants and invariants among the somaclones is not uncommon (Thieme and Griess, 2005).

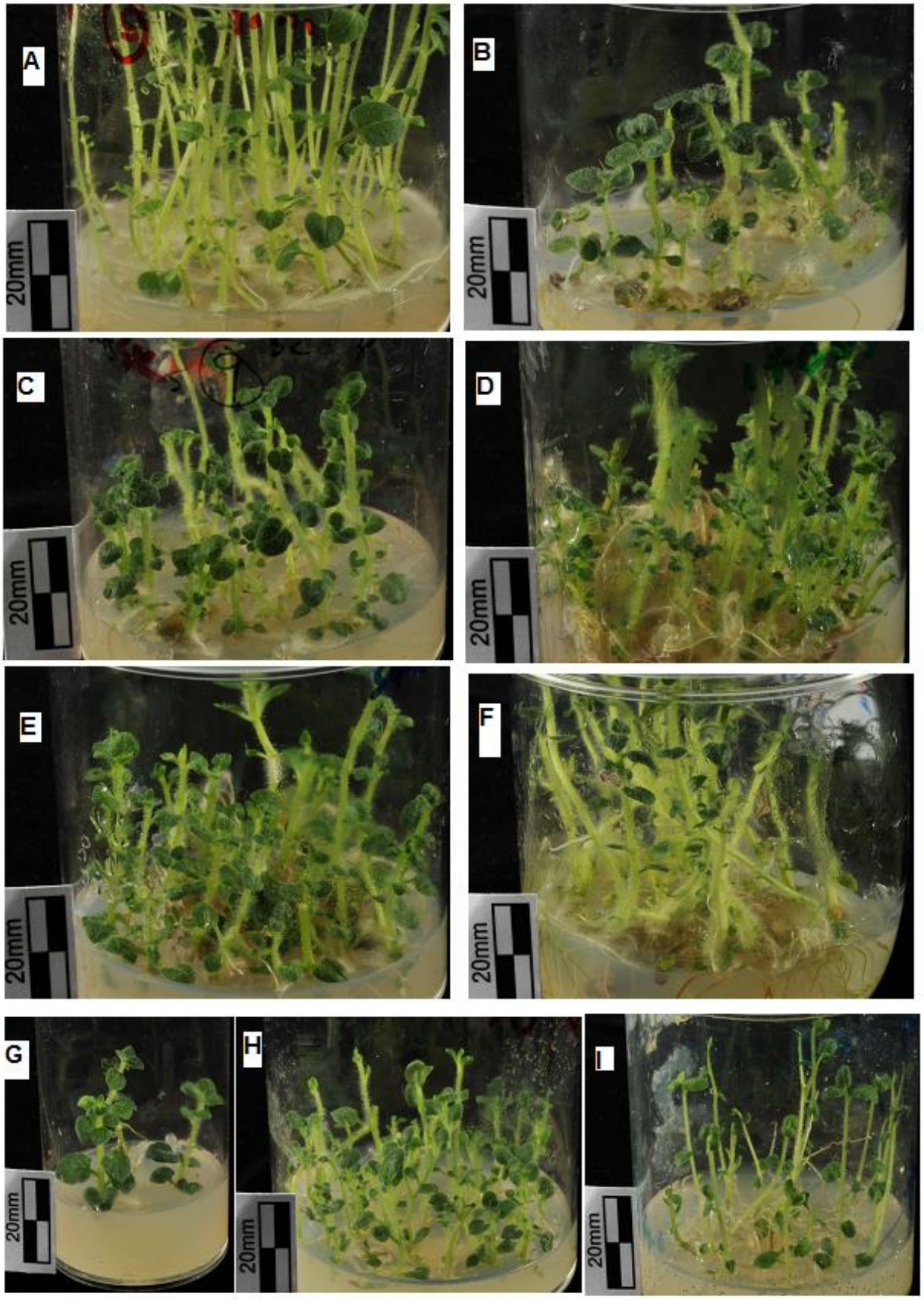


Figure 32. Variations in stem and leaf characteristics of plants regenerated from selected Fe-efficient calli (A-G), callus grown on control medium (H) and plants of the parental biotype (I).

4.5 Summary

An effective protocol for the selection of Fe-efficient somaclonal variants has been developed in potato using callus culture system. The initial visual selection process enabled the quick screening and identification of the desired phenotype (IDC tolerance) over the sensitive calli cells. The establishment of highly repeatable and efficient procedure for plantlet regeneration from callus cultures represents a necessary step in the application of somaclonal variation to enable selection of agronomically desirable traits in potato. The chlorosis tolerant calli were induced to regenerate shoots and root.

Preliminary investigations into callogenesis and plant regeneration revealed that half strength MS medium supplemented with 3.22 µM NAA and 1.78 µM BA was optimal for callus formation while shoot regeneration was most effective on medium supplemented with 6.66 µM BA and 2.89 µM GA_3. The findings show that different concentrations and/ or combinations of plant growth regulators influenced callogenesis and plant regeneration.

The growth habit of all the callus-derived potato plants was erect as the 'Iwa' parent biotype but somaclonal variation leading to phenotypic variations in growth, vigour and leaf characteristics was observed in the regenerants. The regenerants expressed a range of phenotypic variability in terms of size, stem height, thickness and branching, internodal distance, as well as leaf architecture and number. Tall and/or bushy plants, small plants with short internodes, plants with thin, slender or thick stems, plants of small, broad and/or pubescent leaves and plants with numerous or fewer leaves were observed. In all, 35 plant lines were established following micropropagation and bulking up of regenerants. The present work would increase adds to the available information regarding the potential use of somaclonal variation generated *in vitro* to develop novel Fe-deficiency tolerant variants that may be useful for crop improvement in potato and other plants.

CHAPTER FIVE

Characterisation of Fe-efficiency trait in callus-derived plant lines

5.0 Introduction

In vitro selection offers the possibility to manipulate variation by applying selective pressure to obtain variant cell lines and plants of the desired trait. The tissue-culture cycle itself can also induce somaclonal variation (depending on the natural variation in a population of cells) which can be enhanced to attain tolerance to biotic and abiotic stresses (Larkin and Scowcroft, 1981; Brown and Thorpe, 1995). The induction of somaclonal variation and *in vitro* selection have been successfully applied to obtain plants tolerant to different agricultural related stresses. *In vitro* cell culture and plant regeneration is highly beneficial in agriculture with great potential in the control of plant growth, protection of plants against environmental stress, and the production of nutrient enhanced foods. Agriculture is currently shifting from mainly mass production of crops to minimising nutrient loses, maximising plant nutrient use efficiency and focusing on enhancing nutrients in crops.

Effective plant growth on environments threatened by low nutrient content and heavy Fe fertiliser application due to low Fe bioavailability can be made possible through the selection of plants resistant to such conditions. Development of novel Fe-efficient potato variants *in vitro* is beneficial for crop improvement and can be used to complement conventional breeding. The selection and use of Fe-efficient plants is considered the best management approach for alleviating iron-deficiency induced chlorosis (IDC) and for improving crop production (García-Mina et al., 2013; Vasconcelos and Grusak, 2014). Variant cell lines can be isolated by *in vitro* selection and the variant phenotype can persist in regenerated plants (Naik et al., 1990; Thieme and Griess, 2005). To elucidate the stability of a selected trait, the phenotype should be expressed in regenerated plants and be stable in the absence of the selective agent. Plants regenerated from Fe-efficient callus lines were subjected to corresponding Fe-deficiency selection pressures to examine the stability of the trait and to select Fe-efficient plant lines. *In vitro* selection for tolerance to Fe deficiency (Fe-efficiency) trait in potato is valuable given the economic importance of potato and the prevalence of Fe-deficiency worldwide. Selection of novel plant lines with the desirable Fe-

efficiency trait that can thrive in environments deficient in Fe nutrition and enhance the effective use of calcareous soils is important to feed the growing human population.

In this Chapter, the morphological and biochemical responses of regenerants grown under limiting Fe supplies were investigated and plant lines were characterised based on the results attained. Several morphological and biochemical changes in plants regenerated *in vitro* have potential agricultural significance (Vasconcelos and Grusak, 2014). The initial classification of plant lines with respect to their differential tolerance to Fe-deficiency was based on visual chlorosis scores. Morphological attributes of plant lines in relation to leaf, shoot and root characteristics are evaluated. Section 5.4.1 describes and discusses the post regeneration test performed to confirm the Fe-efficiency status of the established plant lines. Section 5.5 presents investigations into the biochemical characteristics of plant lines in terms of photosynthetic pigments content, Fe uptake and antioxidant enzyme activities as well as phenolic contents which influence Fe acquisition under low-Fe availability. The biochemical basis for Fe deficiency tolerance are also discussed.

5.1 Methodology

5.1.1 Test parental (stock) plant cultures under Fe-deficiency conditions

A preliminary experiment was carried out to establish the Fe-efficiency status of the parental biotype by exposing stock plants to a range of Fe-deficiency treatments. Nodal explants excised from stock plants micropagated for 21 days on standard half-strength MS medium of 50 µM iron were transferred to either iron-deficient (0-5 µM) or sufficient (50 µM) medium. Nodal explants were subcultured twice over a period of 28 days. The initial 7-day-old shoots were subcultured into fresh media for 14 days followed by another 7 days of exposure to the corresponding Fe treatments. Intact roots developed were collected for analysis of FCR activity after 7, 21 and 28 days' exposure to Fe deficiency.

5.1.2 Post regeneration testing for IDC tolerance

Regenerants and the parental stock plants were subjected to testing under Fe-deficiency conditions to confirm the phenotype of the Fe-efficiency trait. The main advantage of testing the plant lines is that those tolerant to IDC can be identified and/or confirmed and distinguished from IDC-susceptible plants. All plant lines and control plants (parental stock

plant: SK and plant regenerated from callus supplied with sufficient, 50µM Fe: 50) were assessed. Similar sized plantlets were tested under *in vitro* Fe-deficiency conditions for three successive subcultures over a 3-month period.

Experimental set up for the screening and selection for Fe-efficient plant lines consisted of 12 nodal explants representing 12 biological replicates per plant line and control plants (50 and SK). Each testing regime composed of at most two apical nodal explants per plant line plus the control plants in a 240 ml tissue culture vessel containing 35-40 ml of medium. Plant lines were tested in media with stress levels corresponding to the Fe-deficiency selective pressure (0.005 to 5 µM Fe) under which Fe-efficient callus cells were originally selected. Regenerants derived from IDC tolerant callus cells selected on 0.005 µM Fe medium (A plant lines) were tested on medium of the same Fe content (0.005 µM). Similarly, plant lines B, C, D and E were tested on medium supplemented with 0.5, 0.1, 1 and 5 µM Fe content respectively.

5.1.3 Evaluation of morphological parameters

All morphological parameters were measured after 4-12 weeks of testing plantlets under Fe-deficiency culture conditions. Measurements were done to ascertain the morphological characteristics of the experimental lines in comparison with the control plants. Root, leaf and internode length as well as stem height were measured using a ruler. Leaves were characterised for leaf length, number and colour index (on a 1-5 colour scale). The leaf length measured is the distance from the leaf apex to the base. The length of at least four leaves was measured per plant. Total number of leaves per replicate (plantlet) was counted. Stems were characterised for height and distances between internode. For each plant replicate, the distance between three internodes was taken. There were at least eight replicates per plant line and control plant for all these parameters measured.

5.1.4 Scoring IDC tolerance

Leaf colour was estimated by visual assessment for identification and selection of chlorosis tolerant plant lines. Leaves were visually inspected for differences in leaf chlorophyll pigmentation with the aid of a colour scale. Ratings for the degree of Fe deficiency-induced chlorosis were made by adopting the 1-5 points scale (Abadia et al., 2012; Vasconcelos and Grusak, 2014; Helms et al., 2010). Based on the scale, visual chlorosis ratings (VCR) assigned were 1 = non chlorotic (green leaf), 2 = minor/slight (pale green),

3 = average/moderate (moderate yellowing) and 4 = very chlorotic (intense yellow zones), 5 = extremely chlorotic (severe yellow to yellow-white or pink, bleached). All 35 plant lines were individually examined alongside the control plants. Plants were scored for chlorosis in 2-3 separate experiments. Chlorosis ratings were scored after one and three months' intervals for the 12 replicates cultured per plant line.

5.2 Biochemical analysis

Biochemical parameters (FCR, total phenolics, peroxidase activity, chlorophyll and carotenoid contents) were measured as described in Section 2.6.

5.3 Preliminary findings

5.3.1 Response to Fe-deficiency is dependent of Fe concentration

This experiment was carried out to ascertain the Fe-efficiency phenotype the parental biotype, SK, under the Fe-deficiency treatments used in the original calli selection scheme. This test was necessary in order to find out if the Fe-deficiency selective pressure range (0- 5 µM) used at the cellular (callus) selection level was suitable for inducing Fe-deficiency stress responses in the roots of the Iwa potato plants.

a) Fe-deficiency symptoms in potato stock plants (parental biotype)

The Iwa potato plant under Fe deficiency conditions developed severe to mild leaf chlorosis but plants on control medium showed no chlorotic symptoms (Figure 33). Young leaves of potato plants growing on optimal 50 µM FeNaEDTA medium remained green but the apical young leaves of plants exposed to low Fe concentrations were chlorotic. The severity of chlorosis increased with the level of depletion of Fe in the growth medium (Figure 33): Fe-deprived plants displayed severe leaf chlorosis than plants in 0.5, 1 and 5 µM Fe-containing media. The stems of plants growing on Fe-deficient media were slender while plants with sufficient Fe supplies had thick stems (Figure 33).

b) Root morphology of parental stock plant under Fe-deficiency conditions

Root fresh weight increased in response to rising levels of Fe in culture medium as shown in Figure 34. Plants also developed more roots and gained weight over a prolonged period in culture. The mean root fresh weight of nodal explants cultured for a month in control medium was 2 folds more than that of explants in Fe-starved medium (Figure 34) but the difference was not statistically significant. After three months in culture (Figure 34), mean root fresh weight of plants supplied with sufficient Fe was about 1.5 times greater than that of roots of plants in Fe-starved medium but the difference was not statistically significant. The difference in the mean root fresh weight between the first and after three months of culture was statistically significant at 0 µM ($t= -10.53$, $df= 4$, $p<0.05$) and 50 µM ($t= -6.28$, $df=4$, $p<0.05$).

Plants subjected to Fe-deficiency conditions had shorter roots ($p>0.05$) than plants on sufficient Fe medium for one month. After one month of culture, the length of roots increased by approximately 1 cm comparing plants exposed to Fe-starved (2.83 cm) and Fe-sufficient (3.73 cm) media. Plants grew significantly longer roots after two subcultures (three months) under the same conditions (see Figure 35). The difference in mean root length (2.5 cm) between plants at 0 and 50 µM after three months of culture was not statistically significant ($p>0.05$). The difference in the mean root length after the first and after three months of culture was statistically significant for plants grown on medium containing 0 µM Fe ($t= -5.76$, $df= 4$, $p<0.05$) and 50 µM Fe ($t= -6.76$, $df= 4$, $p<0.05$)

Potato plants growing on optimal Fe medium had roots with few root hairs. On the contrary, roots developed from nodal explants challenged with Fe-deficiency (0-5 µM Fe) had abundant root hairs. Formation of lateral roots and root hairs of potato plants under Fe deficient conditions increased with time. The average length and weight of roots formed under sufficient Fe conditions were about 1 cm longer and twice as much as roots exposed to Fe-deficiency respectively. The number of roots developed from nodal explants cultured at 50 µM were about 27% more than those formed in explants in Fe-free medium.

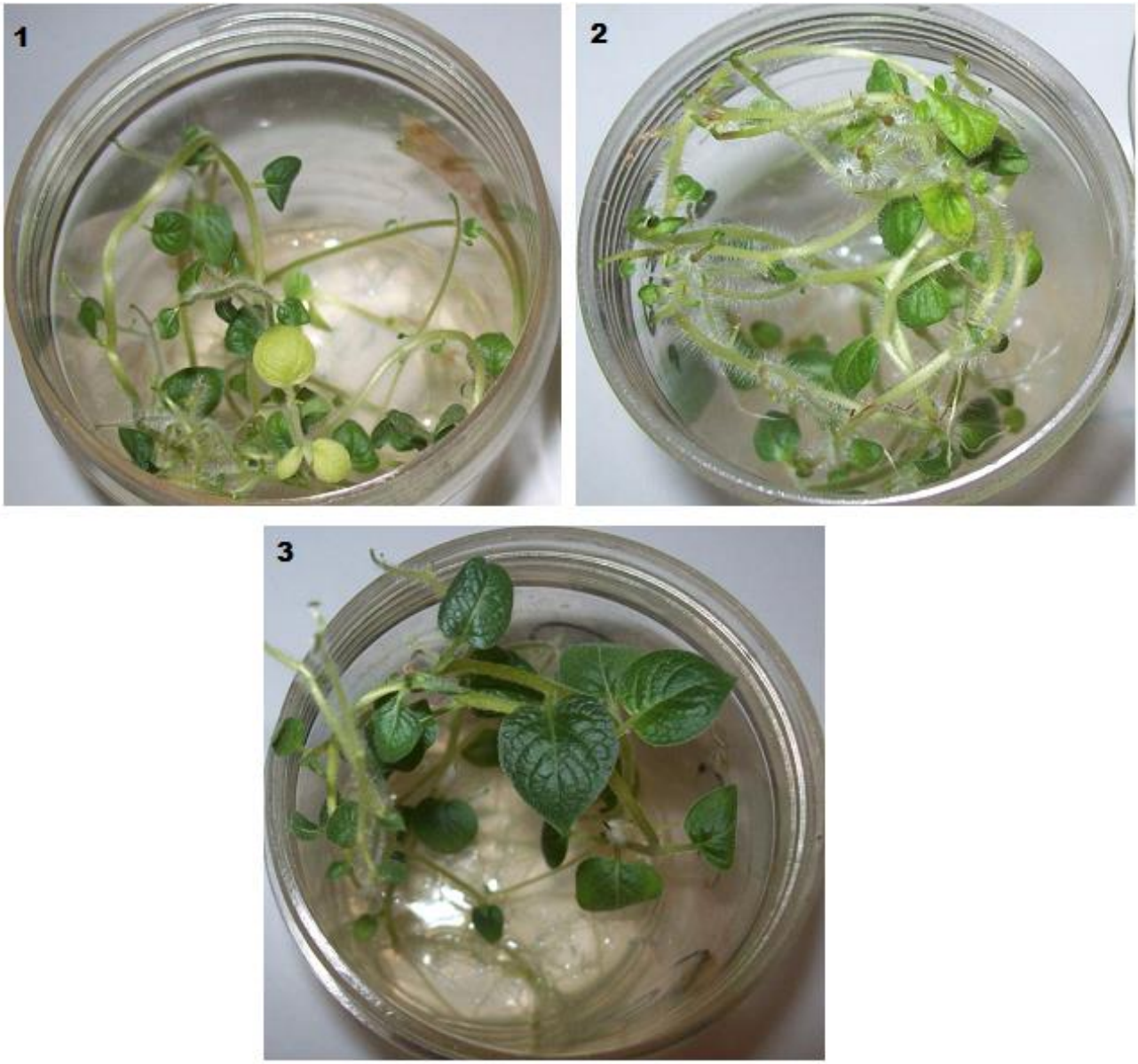


Figure 33. Chlorotic symptoms in Iwa potato stock plants exposed to Fe-deficiency (1: 0 µM, 2: 0.5 µM) and sufficient Fe (3: 50 µM) conditions. Plants were initially grown on medium with sufficient Fe supply (50 µM) and apical nodal explants were transferred to Fe-deficient media and subculture over a two months' duration.

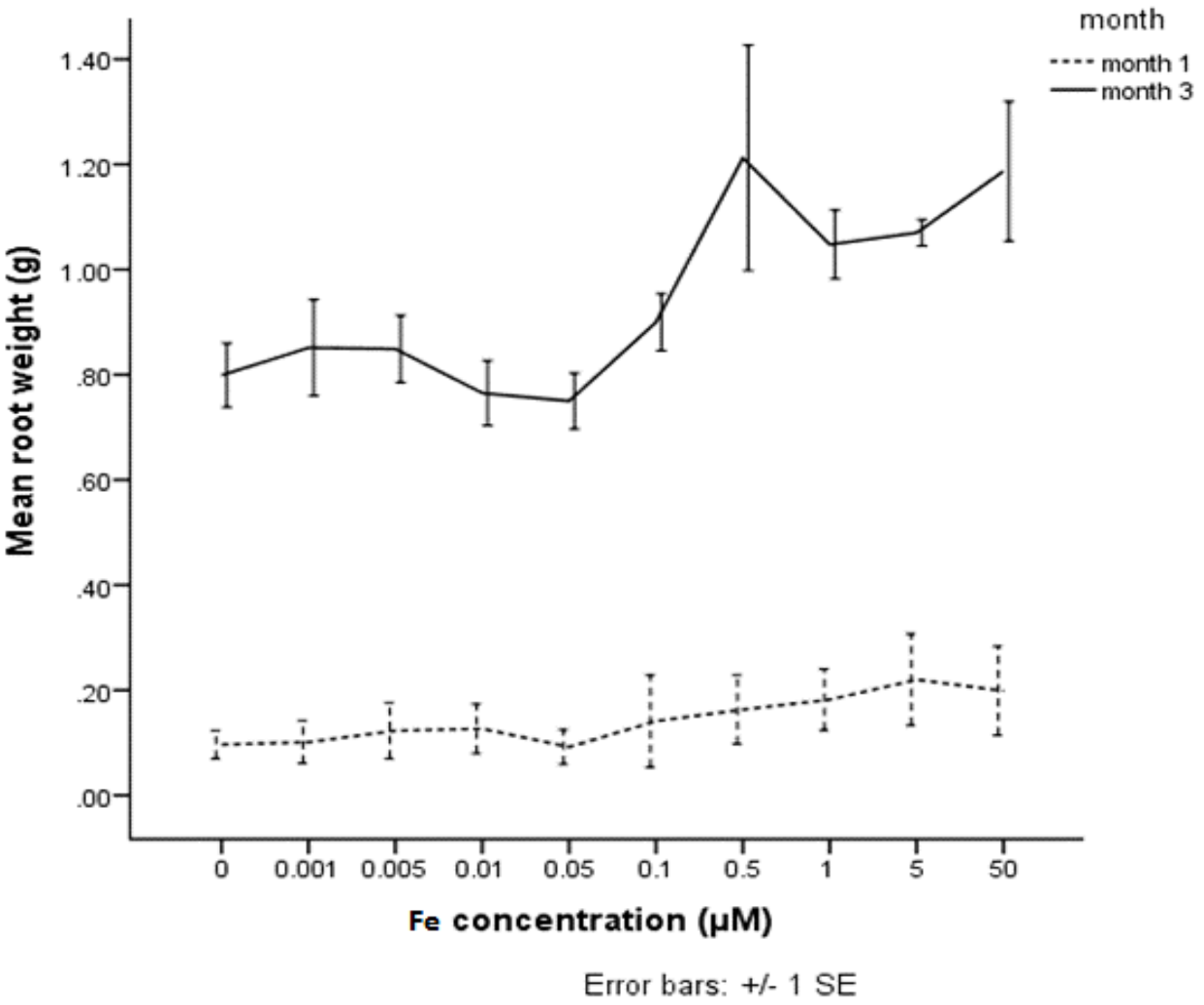


Figure 34. Fresh weight of roots developed from nodal explants cultured in media supplied with different concentrations of FeNaEDTA. Nodal explants excised from plants grown in 50 µM FeNaEDTA half-strength MS medium were cultured in Fe-deficient (0-5 µM) and Fe-sufficient (50 µM) media. Roots were harvested after one and three months' period of culture. Bars represent standard errors (± SE) of the means.

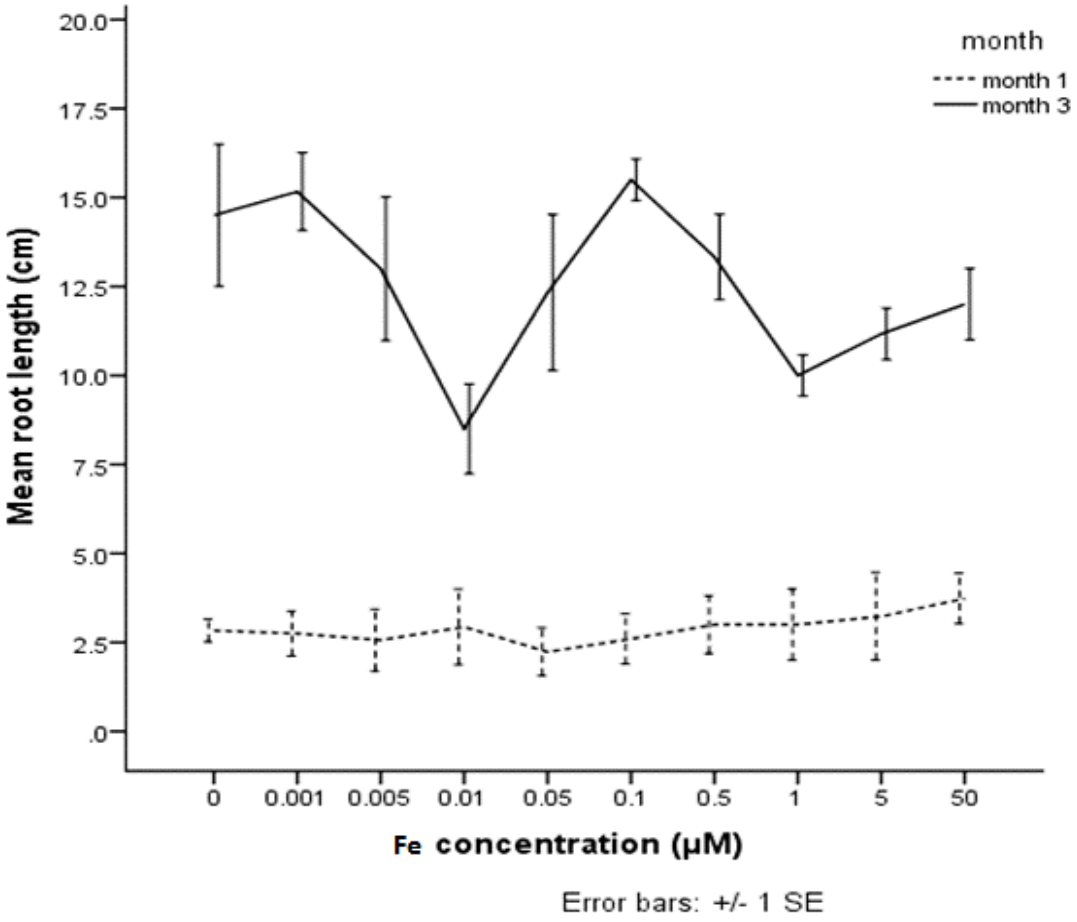


Figure 35. Length of roots developed from nodal explants cultured in media supplied with low and optimal concentrations of FeNaEDTA. Nodal explants excised from plants grown in 50 µM FeNaEDTA half-strength MS medium were cultured in Fe-deficient (0-5 µM) and Fe-sufficient (50 µM) media. Roots were harvested after one and three months' period of culture. Bars represent standard errors (±SE) of the means.

c) Root FCR activity of parental stock plants

The results showed that root FCR activity gradually declined as the amount of Fe in the culture medium increased (see Figure 36). FCR activity was highest in roots grown in Fe-deficient medium while those in Fe sufficient (50 µM) medium showed the lowest FCR activities (Figure 36). The highest activities were recorded in the first week for roots developed from nodal explants cultured 0 and 0.001 µM Fe media.

Alteration in ferric reduction was dependent on the culture duration and the level of the iron deficiency. Iron deficiency caused a significant increase in FCR activity in roots within 7 days. Compared to the exposure to varying Fe concentrations for three and four weeks, elevated root FCR activities were found after one week of culture. The results indicated that FCR activities of two-week-old (see Figure 36 week 3) roots grown in medium

with no Fe (0 µM), low Fe (0.001-5 µM) and optimal Fe (50 µM) media were considerably lower compared to those exposed for a week. Upon exposure to further 14 days (3 weeks from start of subculture) of shoots to low Fe treatments resulted in reduced FCR activity in newly developed roots (Figure 36). When two-week-old roots were removed and the shoots were exposed further to corresponding Fe levels, the FCR activities of the newly grown roots were significantly more and showed a similar pattern to roots developed from old nodal explants after a week of culture. This seems to suggest that Iwa potato roots' response (increase in FCR activity) to Fe supplies was rapid and greatest in the first week of culture.

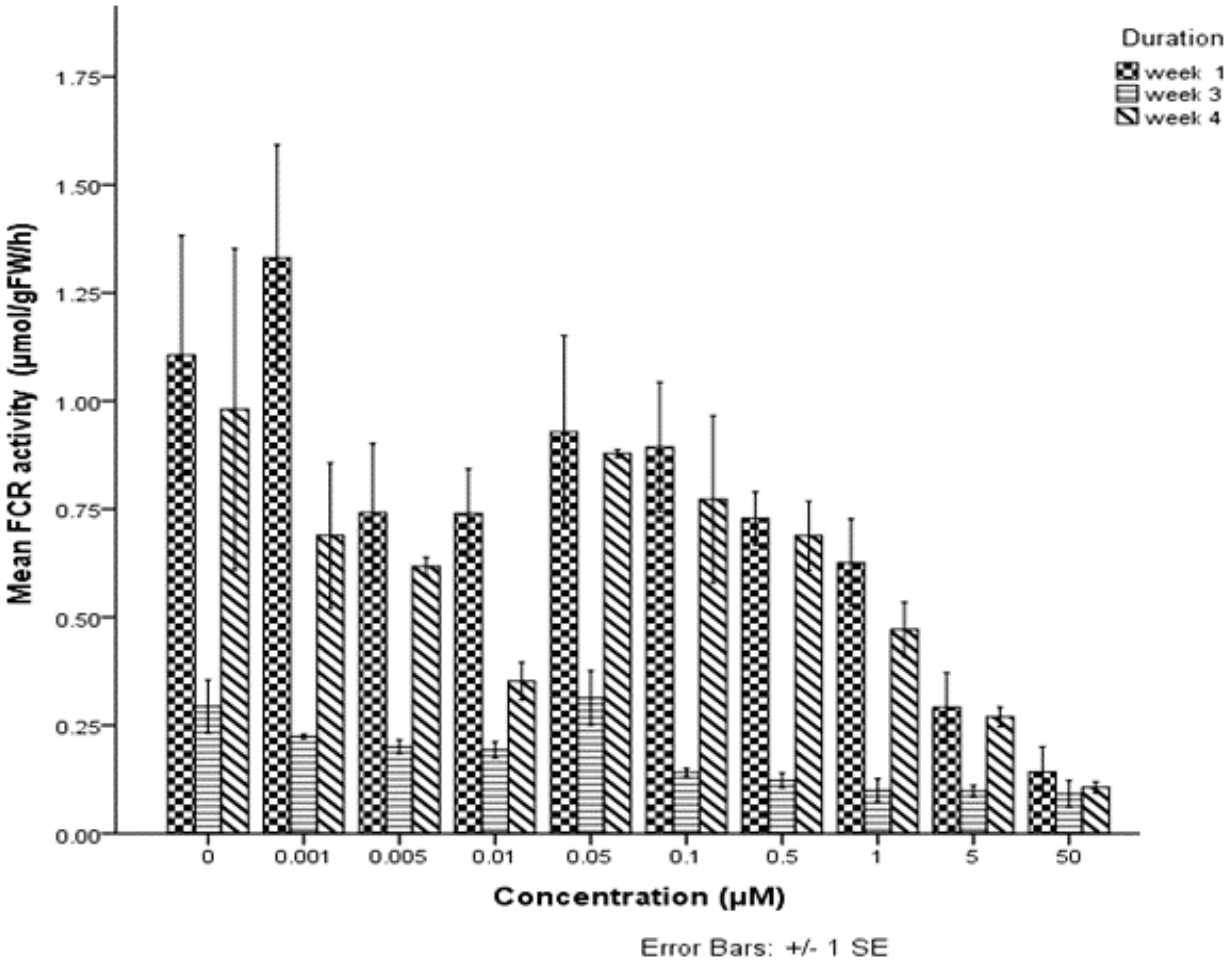


Figure 36. FCR activities in roots developed from nodal explants of Iwa potato stock plant exposed to iron deficiency. Nodal explants excised from plants grown for 21 days on optimal, 50 µM Fe medium were transferred to either iron-deficient (0-5 µM) or 50µM Fe medium. Intact roots were harvested from nodal explants cultured for 7 days (week 1). The 7-day old shoots were subsequently subcultured in fresh media for 14 days (week 3) followed by another 7 days (week 4) subculture in corresponding Fe media. FCR activities of roots harvested in weeks 1, 3 and 4 were determined. Values are presented as means and standard errors (±SE) of the means of four replicates.

The range of Fe concentrations (0-5 µM) used to induce Fe deficiency was found to be suitable in generating the typical Fe deficiency symptoms and responses in Iwa plant leaves (chlorosis) and roots (morphological changes) respectively. Young leaves of the potato plants growing on Fe-deficient nutrient medium developed chlorosis. Chlorosis is reported to occur in plants exposed to Fe deficiency (Abadía et al., 2011; Vasconcelos and Grusak, 2014). The authors observed that iron deficiency caused chlorosis, decreases crop growth, quality and yield. In the present study, root weight and length as well as shoot size were reduced in response to Fe deficiency. Some evidence indicate that other plants showed retarded growth with reduced cell size (decreased shoot and total biomass) when deprived of iron nutrition in growth medium (Tewari et al., 2013).

FCR has been found to be expressed mainly in the epidermal cells of iron-starved roots and is needed by most plants to acquire soluble iron predominantly in response to iron-deficiency (Connolly et al., 2003). Studies focussing on iron reduction at the root level frequently showed that plants had a higher FCR activity under Fe deficiency than under Fe sufficiency. In the present work, root FCR activity was stimulated under limiting Fe conditions but declined with sufficient supplies of Fe. The roots of Iwa potato plants exhibited elevated FCR activity responses to Fe deficiency conditions (0-5 µM). The results strongly support that root FCR activity plays an important role in the response of potato Fe deficiency. The results are in consonance with findings of prior studies on potato (Bienfait et al., 1987), Arabidopsis (Connolly et al., 2003) and pea (Jelali et al., 2010) where FCR activity of roots increased under all conditions of iron deficiency compared to controls. FCR activity was reported to be high in kiwifruit (Rombola` et al. 2002), peach (Molassiotis et al., 2006), and chickpea roots (Mahmoudi et al., 2005) exposed to Fe deficiency conditions. Wu et al. (2012) demonstrated that root Fe(III) reductase activity was activated in a Fe-efficient plant, *Malus xiaojinensis*, upon iron deficiency. The LeFRO1 gene is highly expressed in Fe-deficient roots of tomato (Li et al., 2004) and high PsFRO1 mRNA expression levels were detected in Fe-deficient pea roots (Waters et al., 2002).

Adaptive responses such as modifications in root morphology were observed during culture in Fe-deficient medium. The results herein demonstrated that the development and extent of root hair formation was dependent on the Fe status of the growth medium. Potato plants growing on 50 µM Fe medium had roots with few root hairs. On the contrary, roots developed from nodal explants challenged with Fe-deficiency formed many root hairs. In tomato, root morphological adaptation in Fe-deprived conditions was linked to root hair proliferation (Zamboni et al., 2012). Such morphological responses could have facilitated Fe

uptake processes and/or the activation of FCR synthesis. Regions of the plant roots with lateral roots and dense root hairs were observed to have intense pink pigmentation (see Figure 37) which is indicative of the high/specific sensitivity and direct involvement of the root hairs in the formation of the pink coloured Fe (II)–bathophenantroline disulfonate complex. This pink colouration was low in the roots of plants grown in Fe sufficiency.

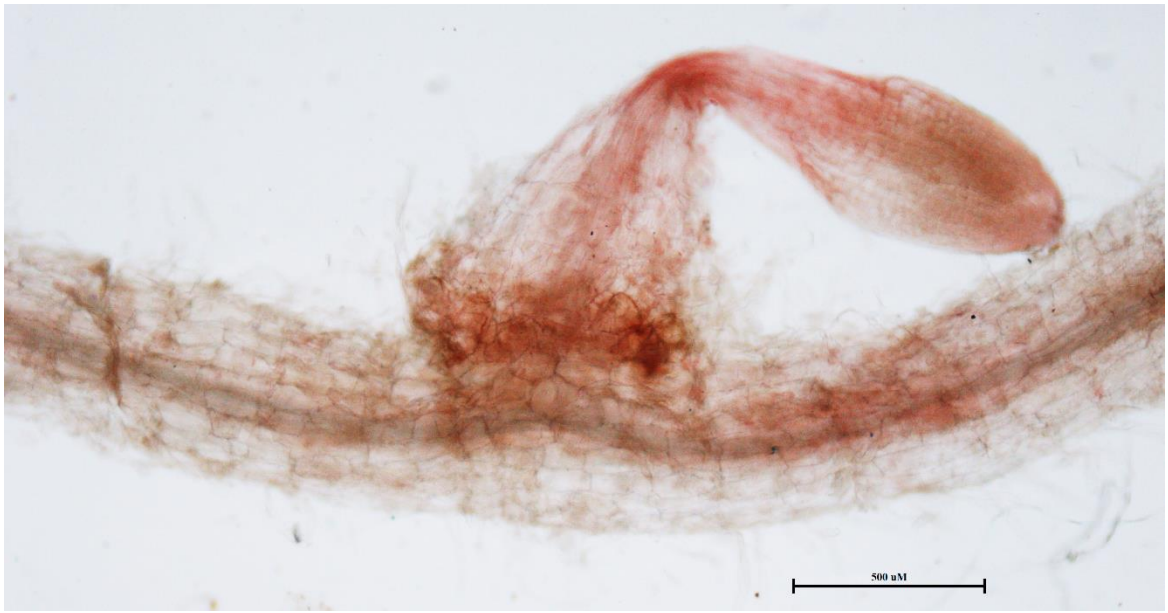


Figure 37. Potato plant root showing intense pink (Fe (II)–bathophenantroline disulfonate complex) pigmentation in mainly lateral root due to high FCR activity. Nodal explants of plants cultured in sufficient Fe medium were transferred to growth medium with limiting Fe supply.

Potato plants growing on control medium had roots with little or no root hairs and a low FCR activity. It is thus suggested that the root hairs play a significant role in the development, response and control of Fe-efficiency reactions in potato plants. The findings of the current study provide evidence that in potato, the roots control iron efficiency response and activates FCR activity as suggested by Bienfait et al. (1987). FCR activity levels were higher in roots with copious amounts of root hairs. Bavaresco et al. (1991) proposed that the presence and amount of root hairs are directly linked to iron reduction. Bienfait et al. (1987) reported on increased root hair formation in roots and high ferric reductase activity in potato plants growing on low Fe medium. According to the authors, potato roots control the development of Fe-efficiency reactions. It appears that the formation of root hairs contributes to adaptation mechanisms under Fe deficient conditions**.** The increased number of lateral

roots and root hairs is assumed to be necessary for a more efficient exploration of the medium. Bienfait et al. (1987) and Wu et al. (2012) demonstrated that the iron-deficiency signal is first perceived in the roots which then trigger responses. The roots possibly develop adaptive mechanisms overtime to enable them survive under Fe-deficiency.

5.4 Main results and discussion

5.4.1 Confirmation of Fe-efficiency trait in callus-derived regenerants

It is not sufficient to assume that if callus cells exhibit Fe-efficiency trait, then the plants derived from them will be Fe-efficient because tolerance can be lost at various levels of plant development. Regenerated plants are required and beneficial in order to elucidate the genetic basis of a selected trait and to confirm phenotype. Owing to the aforementioned, visual ratings for IDC were made (Table 9) subject to growth of regenerants in Fe-deficient medium for one and three months. Leaf chlorosis was evaluated adopting the 5-point visual chlorosis indexing scale (1 = no chlorosis/highly tolerant, 5 = severe chlorosis/susceptible) used in other research (Abadia et al., 2012; Vasconcelos and Grusak, 2014; Helms et al., 2010). The colour chart in Figure 38 was used as a guideline in assigning VCR in order to ensure the consistency of the results.

The results presented in Table 9 show young leaves of virtually all the plant lines were mild or moderate to non-chlorotic after 4 weeks of exposure to Fe-deficiency but a clear difference between plantlets sensitive Fe deficiency (severely chlorotic) and those that are tolerant was observed after 1 to 2 subcultures (2-3 months). A distinction of the differential IDC susceptibility status of plant lines and control plants could mainly be established after prolonged period (3 months) of exposure to Fe-deficient medium. The IDC visual scores for the potato plant lines ranged from 1.00-2.72 after one month on Fe-deficient medium and 1.08 - 4.64 after three months. Within a month of testing plants under Fe deficiency, all of the parental stock plants (control: SK) had low VCR (1.00 to 1.63) within the limit ($\leq$ 2.42) set for assigning a plant as Fe-efficient. After three months however, severe chlorosis was observed in the SK controls with increased VCR ranging from 2.50 to 3.71 and classified a chlorosis sensitive. Regenerated plants derived from callus grown in medium with sufficient Fe supplies had high IDC visual score of 2.63 (in C50) after one-month test in media of

limiting Fe content but markedly higher scores (3.50 - 4.83) after three month's exposure under the same conditions.

Similar to Vasconcelos and Grusak (2014), plants with an average VCR of ≤ 2.42 or ≥ 3.50 were designated as chlorosis tolerant (i.e. Fe-efficient: EF) or chlorosis sensitive (Fe-inefficient: INF) lines respectively (Table 9). The selected EF plant lines had a low combined average chlorosis score of 1.40 ±0.4 (SD) and the INF lines ones had a high score of 3.5 ±0.6 (SD). Susceptibility to IDC was high in the control plants (parental stock plant: SK and regenerants derived from calli supplied with sufficient Fe: 50) and INF plant lines across all the groups of Fe-deficiency treatments (A:0.005 µM -E: 5 µM) used for testing the Fe-efficiency status of the established lines (see Table 9). Regenerants with mean visual chlorosis score less than 1.5 but appeared defective and/or of considerably slow growth rate were not included/selected as promising EF plant lines. Regenerants with moderate-high growth rate and multiplication rates coupled with a high number of leaves produced per plant were selected as putative Fe-efficient plant lines. Using these criteria, 23% (8) EF and 77% (27) INF plant lines were identified (see Table 9). The selected EF plant lines are A1, B2, B9, D1, E1, E2, E3 and E7. All the C lines had mean scores greater than three therefore all were classified as IFN plants.

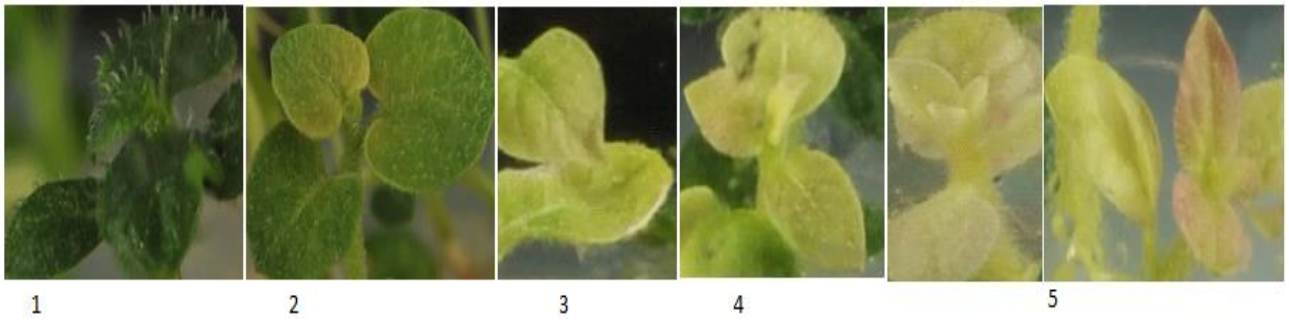


Figure 38. Colour chart showing different degrees of chlorosis in young leaves of potato plants grown in Fe-deficient medium. 1 = non chlorotic (green leaf); 2= minor (pale green); 3= moderate (moderate yellowing); 4 = very chlorotic (intense yellow zones), 5 = extremely chlorotic (severe yellow to yellow-white or pink, bleached).

Chlorosis in young leaves is commonly regarded as the first visual sign of Fe deficiency in plants. The most beneficial strategy that can be used to correct IDC is the selection of chlorosis tolerant (Fe-efficient) plant varieties. Low VCR was used as the criterion for the initial selection for Fe-efficient plant lines (see Figure 39). VCR scores were generally lower in the first month of submitting plants to conditions of low Fe availability compared to two subcultures after (three months). In the first month of testing plants under Fe-deficiency conditions, leaf chlorosis was most prominent in the control plants especially those exposed to 0.005-1 µM Fe medium (A-D). The severity of visual symptoms of IDC-sensitive plantlets consistently increased up to the end of the test period (three months) where some leaves became very yellow, yellow-white or pinkish in colour. IDC tolerant lines were selected after three months of exposure of plants to Fe-deficient media. The selected Fe-efficient plant lines had fewer to no symptoms of IDC and grew better on Fe-deficient medium than chlorosis susceptible (Fe-inefficient lines and controls) plants. Likewise, in *Medicago ciliaris,* Fe deficiency had a more-pronounced impact (severe chlorosis) on IDC sensitive lines than tolerant ones (M'sehli et al., 2014).

Based on the post regeneration IDC tests (see Figure 39), a total of eight plant lines out of 35 plant lines were selected to be potential Fe-efficient lines. The potato lines selected for increased tolerance to IDC are A1, B2, B9, D1, E1-3 and E7. A1 was selected because it showed a consistently lower degree of leaf chlorosis within the three months' exposure to 0.005 µM Fe medium compared to the other A plant lines under the same Fe treatment confirming its relative tolerance to Fe deficiency. Likewise, lines B2, B9, E1-3 and E7. Line D1 did not exhibit chlorotic symptoms in the initial 4 weeks of test on 1 µM Fe medium and had the least mean VCR value among the D plant lines after two test subcultures. Although plant lines E5, E6, E8, E9 and E15 had considerably low IDC scores (1.20 to 1.60), they were not selected as EF lines because they were found have slightly defective morphology such as thin small shoots, fibrous-like stems, no or tiny leaves under Fe-deficiency conditions. Lines designated as INF were identified within each group of plant lines and the severity chlorosis appeared to be cell line-specific and independent of the extent or magnitude of Fe deficiency of the medium.

The chlorosis tolerance trait persisted in the selected EF lines when subjected to long term (3 months) subculturing under Fe-deficiency stress conditions. The stability of all regenerants was tested on return to the selective Fe-deficient medium after growth on non-selective conditions as recommended by Collin and Dix (1990) and Wersuhn et al. (1988). The stability of Fe-efficient potato lines was established by five to six months culturing of the

regenerants in the absence of the selective agent (sufficient Fe medium) and subsequent reintroduction into selective medium.

Chlorosis is a condition that generally refers to a lack of chlorophyll and is associated with a number of abnormalities in plant. Reduced Fe availability especially calcareous soils leads to Fe chlorosis and stunted growth in many crops (Naik et al., 1990; Abadia et al., 2011; Bert et al., 2013). The potato gene that confers phenotype with chlorotic and malformed leaves is denoted *cml* (chlorotic and malformed leaves) (Simko et al., 2008). In the study herein, it is suggested that Fe transport to the leaves may have been impaired in chlorosis sensitive plants (control and INF plants). Jelali et al. (2011) reported that leaf chlorosis can be the consequence of a deficit in Fe^{2+} essential for the biosynthesis of chlorophyll. Iron deficiency affects the structure and function of the photosynthetic apparatus of plant leaves; decrease in photosynthetic pigments arises from the absolute requirement for iron in the formation of thylakoid membrane (Abadia, 1992). Fe^{2+} deficiency can restrict chlorophyll synthesis, induce interveinal chlorosis with necrosis and fall of leaves in the more serious situations), reduce crop quality and yields as well as decrease the nutritional value of edible plant parts (Abadia et al., 2011; Hindt and Guerinot, 2012; Bert et al., 2013; García-Mina et al., 2013).

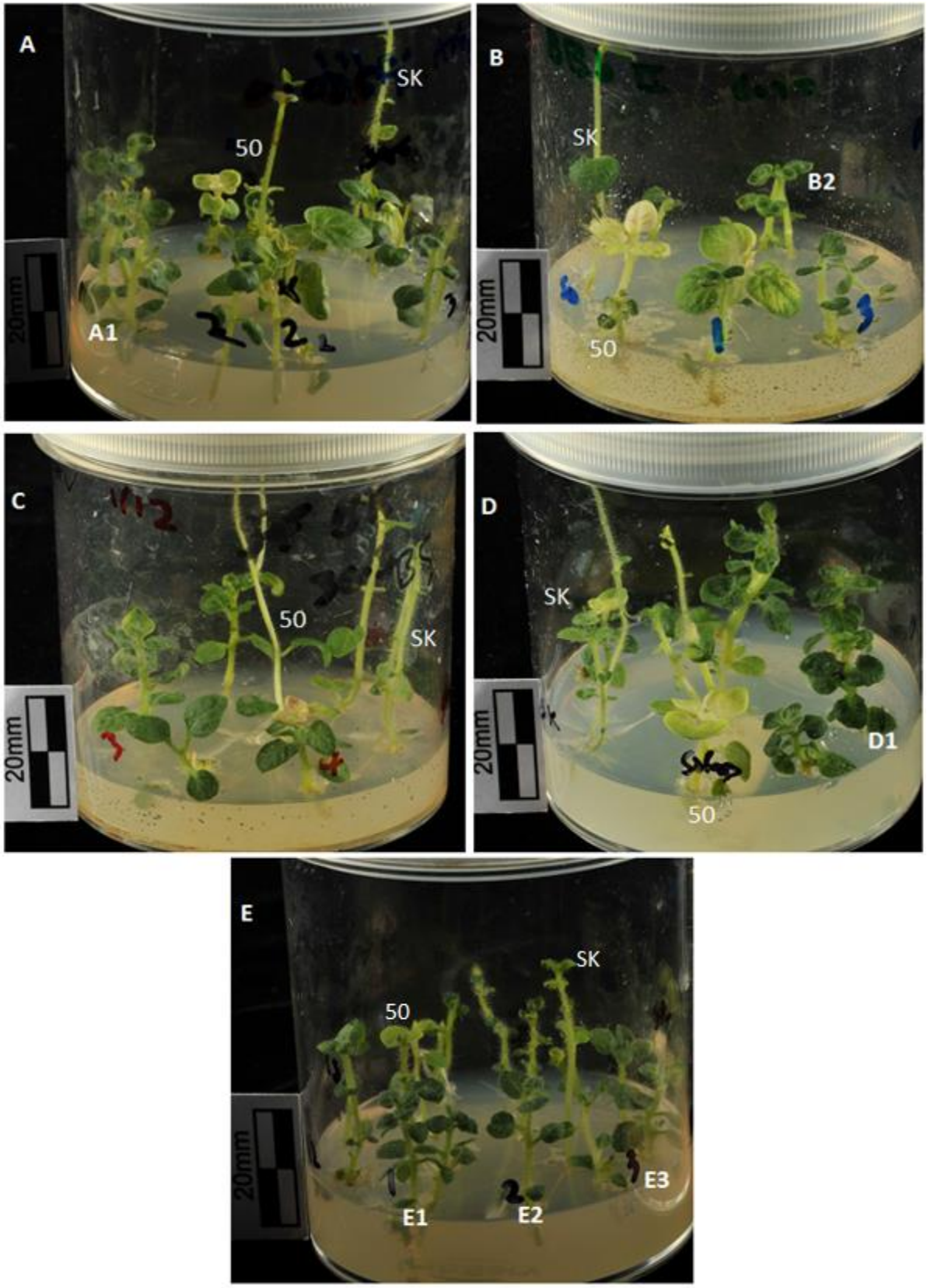


Figure 39. Differential tolerance to Fe deficiency-induced chlorosis among potato plant lines and control plants (SK, 50). Top to bottom: A-E plant lines (regenerated from calli tolerant to 0.005 -5 µM Fe medium) subjected to Fe-deficient medium (A: 0.005 – E: 5 µM). Fe-efficient plant lines indicated are as A1, B2, D1, E1-3.

5.4.2 Morphological characteristics and Fe-efficiency

The data on morphological characteristics of all plant lines and control plants after two months of testing on Fe-deficient media are given in Table 9. The control plants were characterised by tall bending shoot (mostly slender or thin) with lateral stem(s), greater internodal distance, small to medium-sized leaves (see Figure 40 I-II) and high sensitivity to chlorosis. Control plants had a higher propensity to develop lateral stems compared to other plant lines (Figure 40 II). Generally, the experimental plant lines had longer leaves and more leaves per plant compared to the control parental plant, SK. The number of leaves per plant was more in the plant lines than (Figure 40 III-IV) in control plants.

Irrespective of the Fe-deficiency (0.005-5 µM) medium used for screening, the control plants mostly grew significantly taller than the plant lines. Stem heights (in cm) of test plant lines ranged from 23.5 ± 6.15 (in C3) to 76.3 ± 22.6 (in A1). The stem heights of the controls ranged from 72.4 ± 34.1 to 92.6 ± 20.7 cm. The internodal distances of control plants were 2 to 7 times longer those of the plant lines. A proportion of the 35 plant lines were erect with thick short stems, short internodes, had average to broad sized leaves and were either chlorosis sensitive (IFN) or tolerant (EF). Some IFN plant lines (e.g. B15, B16 and C5) showed either enhanced or similar morphological characteristics to that of the control plants (see Table 9): they had slender or thin elongated shoots with long intermodal distances and were severely chlorotic. Contrarily, most EF plant lines had relatively shorter and thick stems with shorter internodes and green leaves. Unlike most EF lines, the A1 line was of tall stem height comparable to some control plants.

Control plants developed significantly longer (2 to 5-folds) and thin green roots with comparatively fewer lateral roots and root hairs (see Figure 41). In all, roots of the plant lines were largely shorter and thicker with few to numerous lateral roots and root hairs (Figure 42). Most EF plant lines had reduced root lengths with greater number of lateral roots than IFN lines (Figure 43). The length and density of root hairs formed in the plant lines appeared to be slightly greater than those developed in control plants (see Figures 42 and 43).

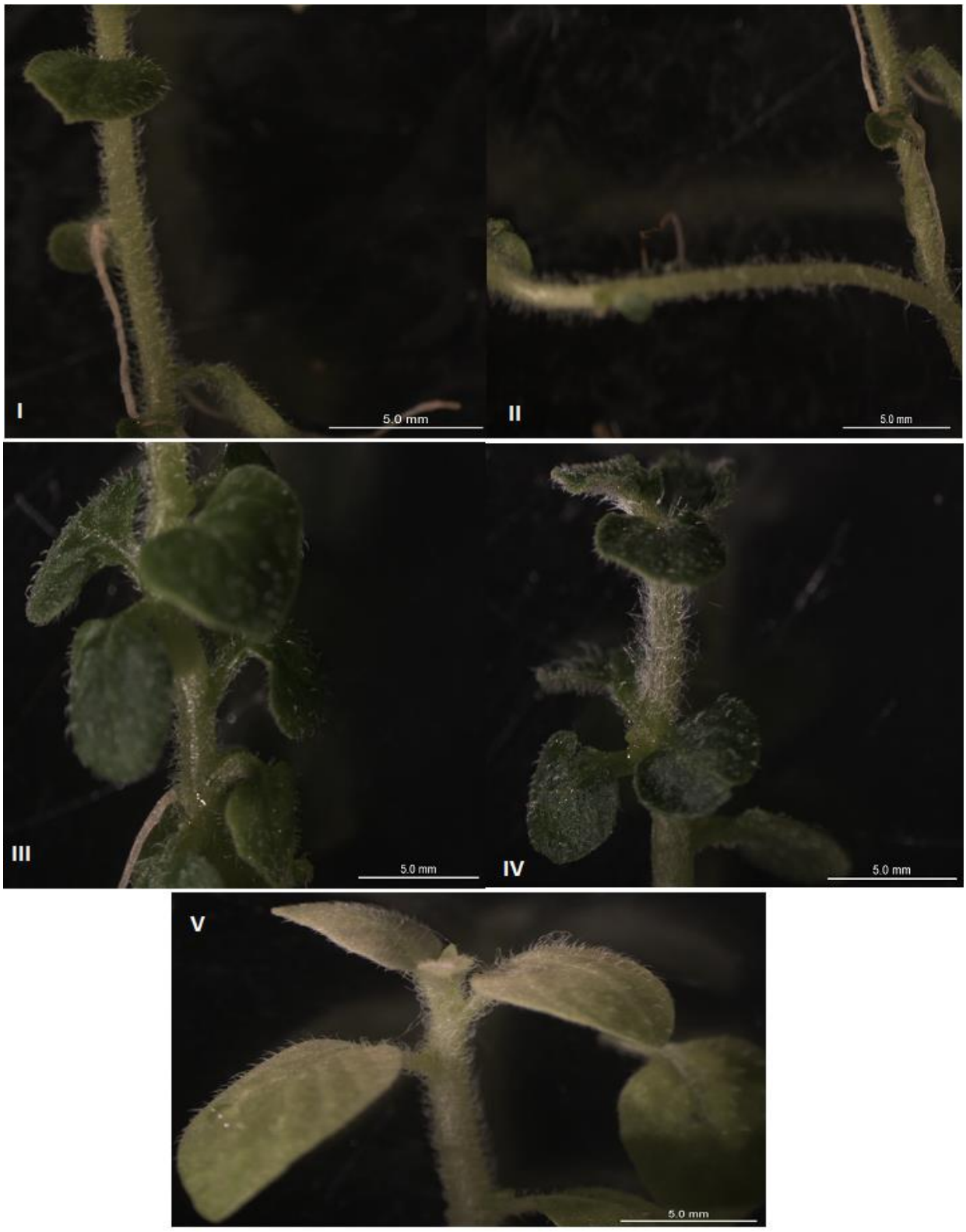


Figure 40. Stereomicroscopic view of morphological characteristics of parental potato stock plants (I, II), and plant lines (II-V) growing under on Fe deficiency conditions. From top to bottom: control plants (I, II) with slender to thin long stems, lateral stem, long intermodal distance, and fewer number of leaves; EF (III-IV) and IFN (V) plant lines with thick short stems, short intermodal distance, numerous average to broad sizes leaves and chlorotic (IFN: V).

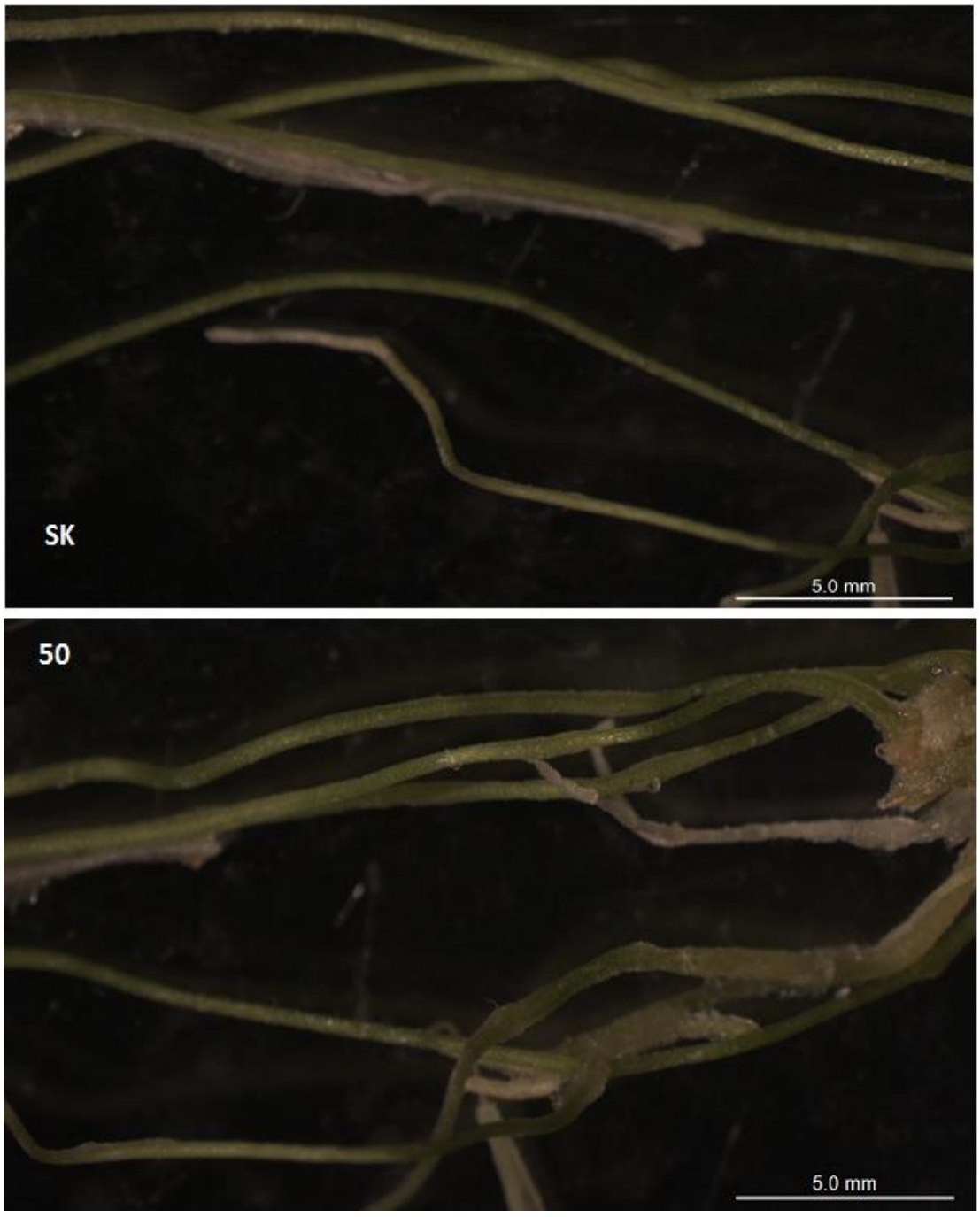


Figure 41. Stereomicroscopic view of roots of control plants (SK, 50) cultured on Fe-deficient medium showing long green roots.

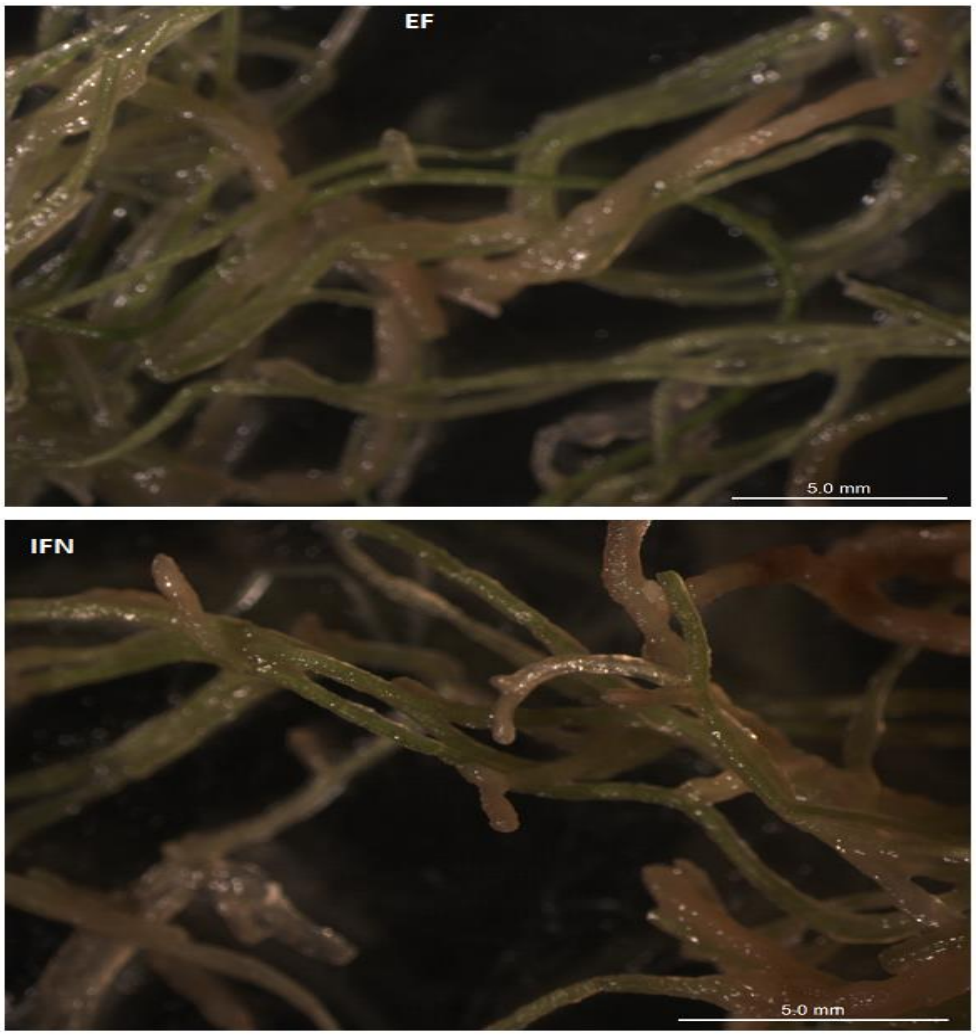


Figure 42. Stereomicroscopic view of roots of Fe-efficient (EF) and inefficient (IFN) potato plant lines on Fe-deficient medium showing numerous short roots most white to cream in colour with some brown regions (in IFN).

Figure 43. Roots of control potato plant (I) and Fe-efficient plant line (II) in Fe-deficient medium showing the development of long roots in control plant and shorter roots with many lateral roots in the EF line (II).

Table 9. Effects of Fe deficiency on chlorosis score, leaf characteristics, intermodal distance, shoot and root length of potato plant lines and control plants (SK and 50). Morphological parameters were measured (n=9) after two-month exposure to Fe deficiency treatments. Chlorosis scores were taken at 1 and three months for all 12 biological replicates per cell line or control plant. Confirmation of IDC status as Fe-efficient (italicised and boldened) or inefficient plant line was based on low mean IDC scores at three months. Control plants are underlined.

cell line	stem height (mm)	internode length (mm)	leaf length (mm)	number of leaves/plant	root length (mm)	Mean chlorosis rating scores 1 month	3 months
A1	76.3 ± 22.6	10.3 ± 3.88	6.03 ± 1.55	13.3 ± 3.50	34.2 ± 11.1	1.08 ± 0.28	1.59 ± 0.70
A2	66.2 ± 28.9	10.7 ± 4.54	4.37 ± 1.06	8.62 ± 3.20	49.8 ± 11.9	1.31 ± 0.48	3.67 ± 1.00
A3	51.2 ± 20.9	8.62 ± 4.13	5.54 ± 1.08	7.08 ± 3.75	39.5 ± 13.2	1.23 ± 0.44	3.50 ± 1.08
A4	60.0 ± 26.6	10.2 ± 5.02	5.23 ± 1.52	9.70 ± 2.75	49.8 ± 14.5	1.50 ± 0.52	3.90 ± 0.88
A50	72.4 ± 34.1	14.1 ± 8.27	5.49 ± 1.91	10.9 ± 3.35	88.8 ± 14.4	2.23 ± 1.18	4.83 ± 0.39
ASK	91.6 ± 18.6	22.8 ± 4.36	5.33 ± 2.77	7.31 ± 1.80	101 ± 26.0	1.69 ± 0.75	3.71 ± 1.11
B1	56.2 ± 30.9	6.67 ± 3.41	5.13 ± 0.36	11.4 ± 2.30	42.4 ± 14.3	1.00 ± 0.00	2.80 ± 0.83
B2	55.0 ± 15.0	7.83 ± 0.44	7.22 ± 2.36	10.0 ± 4.00	35.0 ± 11.8	1.00 ± 0.00	1.38 ± 0.74
B3	38.3 ± 15.8	6.39 ± 3.79	4.86 ± 1.58	10.2 ± 2.23	52.5 ± 17.2	1.33 ± 0.82	2.50 ± 1.03
B4	35.0 ± 13.4	5.42 ± 1.90	4.36 ± 1.25	12.2 ± 2.32	37.7 ± 8.50	1.33 ± 0.52	2.67 ± 1.36
B5	36.7 ± 14.6	4.44 ± 0.19	5.94 ± 1.39	11.3 ± 4.04	43.4 ± 13.7	1.17 ± 0.41	3.20 ± 1.40
B8	45.0 ± 10.0	9.22 ± 3.60	4.72 ± 0.25	8.33 ± 1.53	38.0 ± 10.5	1.33 ± 0.58	3.80 ±1.10
B9	50.0 ± 17.0	7.31 ± 3.86	4.81 ± 1.82	10.3 ± 1.86	35.0 ± 10.5	1.17 ± 0.41	1.33 ± 0.65
B10	39.6 ± 20.9	6.93 ± 2.76	3.50 ± 0.71	6.80 ± 3.03	40.1 ± 9.30	1.00 ± 0.00	3.50 ± 1.64
B12	26.7 ± 5.77	3.67 ± 0.67	4.22 ± 0.38	10.7 ± 5.69	37.6 ± 10.3	2.00 ± 1.00	3.75 ± 1.49
B15	76.7 ± 45.1	14.6 ± 7.64	5.67 ± 1.45	6.33 ± 3.79	70.8 ± 15.6	1.67 ± 0.58	3.00 ± 1.26
B16	75.0 ± 10.0	12.8 ± 1.92	5.22 ± 1.67	8.33 ± 1.52	55.0 ± 10.5	1.00 ± 0.00	4.00 ± 1.00
B50	87.3 ± 17.9	16.7 ± 4.71	5.56 ± 0.38	13.0 ± 2.45	110 ± 22.7	2.60 ± 0.55	4.38 ± 0.92
BSK	84.0 ± 19.8	16.3 ± 5.34	2.07 ± 0.68	6.20 ± 2.77	135 ± 22.3	1.20 ± 0.45	2.53 ± 0.52

Table 9. Continued

cell line	stem height (mm)	internode length (mm)	leaf length (mm)	number of leaves/plant	root length (mm)	Mean chlorosis rating scores 1 month	3 months
C1	33.9 ± 14.1	4.72 ± 1.75	4.78 ± 1.27	14.4 ± 6.13	57.0 ± 10.9	1.50 ± 0.79	4.00 ± 0.95
C2	58.7 ± 36.1	6.42 ± 4.49	4.51 ± 1.61	12.1 ± 4.03	42.8 ± 13.0	1.33 ± 0.65	3.40 ± 1.17
C3	23.5 ± 6.15	2.86 ± 0.63	3.31 ± 1.59	10.2 ± 6.51	53.8 ± 9.30	1.67 ± 0.98	4.00 ± 0.95
C4	33.6 ± 16.8	4.21 ± 2.02	4.85 ± 1.24	12.0 ± 2.65	38.3 ± 16.9	1.18 ± 0.60	3.75 ± 1.06
C5	115 ± 20.1	17.9 ± 4.48	6.09 ± 1.17	11.5 ± 2.42	71.3 ± 20.5	2.72 ± 0.65	4.64 ± 0.67
C50	80.4 ± 27.1	10.0 ± 5.93	5.08 ± 1.00	12.9 ± 3.83	138 ± 25.8	2.63 ± 0.89	4.13 ± 0.99
CSK	92.6 ± 20.7	16.7 ± 3.33	4.13 ± 1.49	9.38 ± 1.99	129 ± 33.1	1.38 ± 0.52	3.50 ± 1.31
D1	44.9 ± 13.6	8.08 ± 1.70	5.74 ± 0.99	14.9 ± 2.90	46.7 ± 13.7	1.00 ± 0.00	2.42 ± 1.24
D2	34.9 ± 11.7	3.87 ± 0.83	5.20 ± 0.97	15.6 ± 2.63	54.5 ± 10.8	1.20 ± 0.42	2.78 ± 1.48
D3	55.9 ± 20.0	7.00 ± 3.73	6.48 ± 1.21	11.8 ± 2.92	52.7 ± 10.0	1.13 ± 0.35	2.63 ± 1.30
D4	32.4 ± 12.3	6.11 ± 4.90	4.52 ± 1.22	14.3 ± 4.15	48.3 ± 14.7	1.33 ± 0.50	3.81 ± 1.47
D50	73.8 ± 30.7	13.7 ± 3.63	5.79 ± 1.85	14.8 ± 3.28	144 ± 19.2	2.00 ± 0.00	3.80 ± 1.14
DSK	74.2 ± 30.9	8.64 ± 5.07	3.39 ± 1.68	8.50 ± 3.21	146 ± 33.9	1.00 ± 0.00	2.75 ± 0.62
E1	54.5 ± 15.3	6.89 ± 0.97	5.61 ± 2.39	16.5± 6.90	44.2 ± 11.1	1.00 ± 0.00	1.08 ± 0.29
E2	53.8 ± 15.4	9.31 ± 4.92	6.08 ± 1.94	14.7 ± 5.46	50.0 ± 14.4	1.00 ± 0.00	1.13 ± 0.34
E3	43.5 ± 23.0	7.97 ± 1.48	6.11 ± 1.07	13.3 ± 6.53	90.0 ± 25.3	1.00 ± 0.00	1.17 ± 0.39
E4	45.7 ± 26.1	8.44 ± 1.34	6.56 ± 1.17	15.3 ± 4.93	39.2 ± 16.3	1.00 ± 0.00	3.63 ± 1.40
E5	52.0 ± 26.7	6.29 ± 2.86	6.21 ± 1.23	11.3 ± 2.36	44.2 ± 12.4	1.25 ± 0.50	1.60 ± 0.92
E6	58.3 ± 12.6	6.50 ± 1.17	4.00 ± 0.58	13.7 ± 2.31	32.5 ± 10.8	1.00 ± 0.00	1.50 ± 0.53
E7	39.6 ± 22.2	6.23 ± 4.58	3.67 ± 0.94	11.8 ± 4.09	43.3 ± 10.3	1.00 ± 0.00	1.13 ± 0.34
E8	60.0 ± 5.00	8.78 ± 2.52	3.33 ± 1.20	11.0 ± 1.00	30.7 ± 7.3	1.00 ± 0.00	1.20 ± 0.00
E9	56.7 ± 11.5	11.6 ± 0.92	3.22 ± 0.19	8.00 ± 4.36	39.2 ± 15.9	1.00 ± 0.00	1.25 ± 0.25
E12	36.7 ± 14.4	8.67 ± 1.20	1.67 ± 0.78	6.00 ± 2.65	32.5 ± 10.8	1.00 ± 0.00	2.00 ± 0.50
E15	68.3 ± 15.3	10.0 ± 2.50	6.67 ± 1.33	12.7 ± 1.53	40.8 ± 14.3	1.00 ± 0.00	1.20 ± 0.20
E50	75.0 ± 15.4	11.2 ± 9.05	7.27 ± 2.62	11.8 ± 6.18	159 ± 34.0	1.63 ± 0.44	3.50 ± 1.29
ESK	84.2 ± 12.4	19.2 ± 4.05	3.91 ± 1.71	7.86 ± 1.77	150 ± 37.4	1.43 ± 0.53	2.50 ± 1.20

The morphology of plant lines and the control parental biotype were assessed in order to study the influence of Fe-deficiency stress conditions. Fe-deficiency adversely affects plant growth and is cultivar-dependent (Jelali et al., 2011). In response, plants undergo morphological alterations to enhance Fe uptake capacity (Broadley et al., 2012; Zamboni et al., 2012). Additionally, plants adjust their relative biomass allocation and distribution to roots or shoots under nutrient limiting pressures or conditions, a phenomenon known as allocation plasticity. Variations in biomass allocation patterns are indicative of how plants respond to different selection pressures and /or environmental stress.

Usually there is a cost in fitness to selection. A potential loss of yield and/or competitive ability following selection for a trait is referred to as loss of fitness (Haldane, 1960). Slower growth is an adaptive feature for plant survival under stress and the extent of tolerance is suggested to be inversely related to growth rate (Queiros et al., 2007). Changes in whole plant growth and development relating to Fe deficiency have been reported: plant development was considerably restricted by the Fe deficiency induced treatment (Mahmoudi et al., 2009). Suboptimal Fe levels adversely affected whole plant dry weight but short term exposure to Fe deficiency did not affect whole plant dry weight and shoot length (Mahmoudi et al., 2009; Jelali et al., 2011). Some evidence indicates that plants show retarded growth with reduced cell size (decreased shoot and total biomass) when deprived of iron nutrition in growth medium (Tewari et al., 2013; Chaterjee et al., 2006). In accordance with the findings of the study herein, Fe-efficient soybean plants were observed to generally be the shortest compared to inefficient ones (Elmstrom and Howard, 1969; Vasconcelos and Grusak, 2014). Similarly, the stem height of both chlorosis-sensitive and tolerant chickpea varieties exposed to Fe deficiency was markedly lower than the respective controls (Mahmoudi et al., 2009). Under Fe deficiency treatments, shoot height and fresh weight and growth were reduced significantly in chlorotic compared to non-chlorotic plants (Kabir et al., 2015; M'sehli et al., 2008).

Morphological variations in leaf development and size have been linked to Fe deficiency chlorosis. Chlorotic leaves have significantly reduced leaf expansion, size, fresh and dry weight (Larbi et al., 2006; Fernández et al., 2008) but increased leaf thickness (Maldonado-Torres et al., 2006) as compared to non-chlorotic leaves under Fe-sufficient conditions. Fernández et al. (2008) proposed that IDC can prevent leaf development via changes in leaf cuticle and cell wall. They indicated that green leaves have thick and homogeneous cell walls while cells in chlorotic leaves appear as thin, discontinuous and

heterogeneous. Also, the number of stomata per leaf was decreased in chlorotic leaves which affects stomatal functioning. Fe-deficiency can modify the barrier properties of the leaf surface, which can have a marked effect on leaf water relations, solute permeability and pest and disease resistance (Fernández et al., 2008). Fe shortage results in reduced leaf growth, leaf number, leaf surface area, leaf biomass, loss of turgor and the inhibition of the formation of new leaves (Kabir et al., 2015; Kosegarten and Koyro, 2001; Mahmoudi et al., 2009). Poor leaf growth and suppressed leaf formation are characteristic symptoms of IDC response due to the high sensitivity of the meristematic apex to low iron availability (Gruber and Kosegarten, 2002; Vasconcelos and Grusak, 2014).

Fe deficiency-induced morphological and physiological modifications in plant roots are dependent on the plant species (Broadley et al., 2012). In strategy 1 plants (e.g. potato), Fe-deficiency is associated with inhibition of root elongation, increase in diameter of apical roots, subapical swelling with abundant root hairs and formation of transfer cells (Broadley et al., 2012). The most efficient way of increasing absorbing root surface area is in the formation of root hairs. In the current study, the increased number of lateral roots and root hairs identified in the potato plant lines especially the Fe-efficient ones are proposed to be necessary for a more efficient exploration of the medium for the acquisition of Fe nutrient. Also, most EF plant lines had reduced root lengths than IFN lines contrary to previous reports in other plants. Increase and decrease in root biomass was observed in *Medicago ciliaris* chlorosis tolerant and sensitive lines respectively (M'sehli et al., 2008). Similarly, a significant decline in root length fresh weight as root and root growth was observed in a chlorosis-susceptible okra variety compared to a tolerant variety under low Fe availability (Kabir et al., 2015). Although root biomass was unaffected by Fe in medium, reduction in dry weight and root biomass was more severe in chlorosis-susceptible chickpea varieties than tolerant ones in comparison to their respective controls under bicarbonate-induced Fe deficiency (Mahmoudi et al., 2009). Fe-deficient plants developed more lateral roots and abundant root hairs (Licciardello et al., 2013). Improvement in root hairs formation in Arabidopsis mutant was regulated by the Fe concentration of the growth medium (Schikora and Schmidt, 2001). Fe deficiency-induced formation of rhizodermal transfer cells is a part of mechanism for enhancing Fe uptake (Romheld and Marschner, 1981; Broadley et al., 2012). Excretion of proton, reducing capacity and release of phenolics occur at transfer cell sites (Broadley et al., 2012). In the present study, differences (increased lateral roots, root hairs and decreased length) observed in the root morphology of plant lines (especially Fe-efficient lines) could be pivotal for the development of potato cultivars with potential for enhanced Fe

uptake. In tomato (Zamboni et al., 2012) and potato (Bienfait et al., 1987) root morphological adaptation under suboptimal Fe conditions was linked to enhanced root hair proliferation. When plants growing under Fe deficient conditions were resupplied with Fe, morphological root responses were diminished and transfer cells degenerate (Broadley et al., 2012).

Iron deficiency-induced chlorosis is a variable trait that is influenced not merely by environmental conditions, but correspondingly by genetically determined factors, inherent in the cultivar itself (Vasconcelos and Grusak, 2014). Due to the differences in the *in vitro* Fe-deficiency culture (0.005 to 5 µM Fe) used to select chlorosis tolerant calli, and subsequently to test regenerants, the different morphological responses might not solely be as a result of the suboptimal Fe supplies but can also be due to the somaclonal variation resulting in distinctive individual plant lines each with unique morphological characteristics.

5.4.3 Test for relationship and associations between morphological characteristics of cell lines

The number of leaves per plant is significantly negatively correlated with internodal distance but positively correlated with leaf length as shown in Table 10. This gives an indication that plant lines with shorter internodal distances had a higher number of leaves and longer leaf length. The number of leaves was significantly increased with a decrease in internodal length. Most EF plant lines and some IFN lines displayed the aforementioned characteristics. The correlation between IDC scores, number of leaves, leaf and internode lengths was very weak.

Enhancement in root length was significantly related to increase in stem height as was characteristic of the control stock plants. Plant root length appeared to contribute to IDC susceptibility (control and IFN plants) since there was a significantly positive correlation between VCR score and root length. Stem height had a highly positive relationship with intermodal distance, leaf and root lengths. This implies that plant lines (mostly IFN lines) and control plants that were taller had longer roots, leaf length and intermodal distances as summarised in Table 10. Fe-efficient soybean plants were identified to be the shortest and this was confirmed by the negative correlation found between stem length and IDC tolerance (Elmstrom and Howard, 1969).

The results in Table 11 present a significantly positive association between control plants and the likelihood of having a high chlorosis score (chlorosis sensitive) with increased stem height, root and internode length upon exposure to Fe-deficiency conditions. A

unit increase in chlorosis score, stem height, root and internode length would raise the odds of a plant being a control plant (SK or 50). Odds ratio for leaf length, number of leaves per plant was less than 1 (see Table 11) suggesting that these characteristics had a low likelihood of being associated with control plants exposed to low Fe medium. It can be inferred that such characteristics could be associated with the selected potato plant lines. There was a strong link between control plants and IFN plant lines and the likelihood of susceptibility to chlorosis (high IDC score: chlorotic).

Table 10. Spearman's correlation coefficients for visual chlorosis scores and morphological parameters measured in potato plant lines and control plants. Plant materials were collected and assessed after two-month exposure to Fe-deficiency conditions. The dataset was obtained from the analysis of all the different potato plant lines and control plants.

Parameter	Stem height	Internode length	Leaf length	Number of leaves/plant	Root length	Visual chlorosis ratings
Stem height	1.00	**.71****	**.17****	-.04	**.35****	-.03
Internode length	**.71****	1.00	.09*	-.14**	.30**	.01
Leaf length	**.17****	.00*	1.00	.25**	.07	.06
Number of leaves/plant	-.04	**-.14****	**.25****	1.00	-.07	-.11
Root length	**.35****	**.30****	.07	-.07	1.00	**.30****
visual chlorosis ratings	-.03	.01	.06	-.11	**.30****	1.00

**. Correlation is significant at the 0.01 level
*. Correlation is significant at the 0.05 level

Table 11. Odds ratio, Exp(B), values for the association of morphological characteristics of plants and exposure to Fe-deficiency. Control plants and plant lines were cultured in Fe-deficient medium for two-month.

Morphological parameters	Exp(B)	Sig.
Chlorosis	1.70	0.00
Root	1.13	0.00
Number of leaves/plant	0.90	0.00
Leaf length	0.97	0.59
Internode	1.26	0.00
Stem height	1.04	0.00

5.5 Biochemical mechanisms involved in Fe-efficiency

Selection of chlorosis tolerant plants solely based on their IDC scores and morphology should be followed by further studies to obtain a deeper knowledge of the tolerance mechanism(s) involved. Morphological characterisation may not be an exact mirror of genetic change because plant morphology is usually influenced by environmental factors (Bairu et al., 2011). Therefore, the biochemical aspects which make a certain plant or cell line more IDC tolerant needs to be investigated to delineate the biochemical mechanisms associated with such a trait. Plants can be classified as Fe-efficient if they respond to Fe deficiency stress by inducing biochemical reactions that make Fe available in a soluble form and as Fe-inefficient, if they do not (Vasconcelos and Grusak, 2014; García-Mina et al., 2013). It is necessary to decipher the biochemistry of tolerance and sensitivity to iron deficiency in order to biochemically characterise potato plant lines as EF or IFN. This section presents the results of various biochemical tests performed on the established potato plant lines and discusses the similarities and differences between EF and IFN plant lines and control plants (SK: parental stock plant; 50: regenerant plants derived from calli grown on 50 µM Fe medium).

5.5.1 Chlorophyll involved with IDC tolerance

a) Leaf chlorophyll content

The leaves of putative Fe-efficient plant lines had significantly higher ($p<0.05$) chlorophyll content than IFN lines and control plants cultured in a medium of the same Fe-deficiency level (see Figure 44). Also, leaf chlorophyll content was higher in EF than IFN lines and control plants irrespective of the Fe-deficiency level of the medium for example A1 compared to C5, D4, E4 (see Figure 44). That is A1 (EF line) on 0.005 µM Fe medium had more leaf chlorophyll content than IFN lines C5 (on 0.1 µM Fe medium), D4 (on 1 µM Fe medium) and E4 on (5 µM Fe medium). Out of the eight selected Fe-efficient plant lines, 75% (A1, D1, E1-3 and E7), exhibited the highest mean leaf chlorophyll content ranging from 103-175 mg/mL and the other 25% (B2 and B9), had values from 80-92 mg/mL.The mean difference in leaf chlorophyll content among 75% of the plant lines, the control plants and highly chlorotic lines (A2-4, B8, B5, B12, C3, C5, D4 and E4) was statistically significant ($p<0.05$).

The mean total leaf chlorophyll content in the selected Fe-efficient line, A1, was significantly greater than in IFN lines A2 and A3 by 17% and 43% respectively and 67 mg/ml more than in plant line A4 all cultured under the same Fe-deficiency conditions. The mean difference in leaf chlorophyll content between A1 and the controls was 88 mg/mL (ASK) and 66 mg/ml (A50), respectively. About 30% of the B plant lines had significantly increased ($p<0.05$) total leaf chlorophyll values relative to that of control plants. The B2 and B9 Fe-efficient lines had the highest mean total leaf chlorophyll content among the B plant lines. The mean amounts of chlorophyll in B2 and B9 lines was about 1.5 to 3 folds greater than the controls BSK and B50, respectively. The mean leaf chlorophyll levels in 70% of the IFN B lines (mostly highly chlorotic) were lower than the EF lines by an average of 35-70 mg/ml. Interestingly, the B12 Fe-inefficient line had the least leaf chlorophyll content with levels about four times less than the EF lines and 1.5 to 3 times less than BSK and B50 control respectively.

All the C lines were designated as IFN since their mean VCR scores were greater than three. The highly chlorosis-susceptible line, C5 (with VCR of 4.64 ± 0.67) had the least amount of total leaf chlorophyll content (16.3 mg/ml) among all plant lines and controls evaluated. Leaf chlorophyll content of 60% of the C lines was significantly decreased by 1 to 6-folds compared to the control plants (CSK, C50) but there was no significant difference ($p>0.05$) in the mean leaf chlorophyll between C2, C4 and the controls (Figure 43).

Generally, the mean leaf chlorophyll content in the D lines EF line (D1), IFN lines (D2 and D3) and DSK control was similar however, leaf chlorophyll content in the D4 Fe-inefficient line (of VCR 3.81 ± 1.47) was markedly declined by 50-54% relative to other D lines and the DSK control ($p>0.05$).

The chlorophyll content in leaves of plant lines tested on E (5 µM Fe) medium was largely more than those tested on media with Fe levels 1000-10 times lower (A-C). The mean leaf chlorophyll levels in 50% of the E lines were equivalent (110 mg/ml). However, selected EF plant lines E1 and E7 had 1.2 to 4.5-folds more leaf chlorophyll content than the control plants and other E plant lines. Surprisingly, leaf chlorophyll content measured in E3 and E2 potential EF plant lines was 8% and 15% less ($p<0.05$) respectively compared to ESK but increased by 46 -51% relative to the E50 control ($p<0.05$). The ESK control was less chlorotic (VCR score =2.50 ±1.20) on E (5 µM Fe) medium and had leaf chlorophyll content of about 4 times more than that of the chlorosis sensitive E50 control (VCR score = 3.50 ±1.29).

Photosynthesis (associated with chlorophyll levels) is considered a physiological indicator or biomarker of plant tolerance to stress since plants reduce their photosynthetic rate as a first response to unfavourable environmental conditions (M'sehli et al., 2014). Low chlorophyll content (chlorosis) of young leaves is the most evident sign of Fe-deficiency because Fe plays a critical role in chlorophyll biosynthesis. Fe is needed for production of proto-chlorophyllide from Mg-protoporphyrin (Pushnik and Miller, 1989). Chlorosis is linked to a loss of chlorophyll content and is associated with modifications in the expression of certain components of the photosynthetic apparatus (Donnini et al., 2009; Larbi et al., 2006). During Fe scarcity, chloroplast volume and protein content decline implying that the protein-chlorophyll complex of chloroplasts is not synthesised adequately thus, leading to chlorosis (Nikolic and Romheld, 2007). Chloroplasts and thylakoids are sensitive to Fe-deficiency because Fe is essential for chlorophyll biosynthesis (Broadley et al., 2012; M'sehli et al., 2014). Previous studies have implicated iron shortage in the reduced formation of chlorophyll. For example, a substantial decrease in chlorophyll content and net photosynthesis in leaves of plants grown in medium with low Fe concentration has been reported (Iturbe-Ormaetxe et al., 1995; Ojeda et al., 2005). Also, Fe deficiency was shown to reduce chlorophyll contents in legume (M'sehli et al., 2009; Jelali et al., 2011) and chickpea (Mahmoudi et al., 2007).

In the current study, the total chlorophyll content, which is related to photosynthetic activity, revealed differences in Fe deficiency tolerance among potato plant lines and control plants. The specific differences identified between plant lines and control plants in relation to leaf chlorosis under low Fe supplies may be explained by the decrease in chlorophyll status, predominantly in the sensitive lines and controls compared to tolerant lines. Chlorophyll levels in chlorosis-susceptible control plants and IFN lines were largely low under Fe-limiting conditions. Reduction in leaf chlorophyll content has been attributed to the regulatory role of Fe in the formation of aminolevulinic acid and protochlorophyllide, the precursors of chlorophyll biosynthesis (Jelali et al., 2011; Broadley et al., 2012). Increase in chlorophyll concentration and a decrease in chlorosis symptoms appear to be associated with Fe-efficiency. This implies that determination of chlorophyll levels could be effectively employed for rapid screening of iron deficiency tolerance in potato. In an earlier study, selected Fe-efficient plantlets (regenerated from Fe deficiency tolerant callus) were found to have nearly 4-folds more chlorophyll content than inefficient plants (Naik et al. 1990). Similarly, IDC tolerant soybean plants showed higher chlorophyll levels under Fe-limiting conditions (Vasconcelos and Grusak, 2014). According to the authors, soybean plants categorised as IFN had low chlorophyll content compared to EF cultivars in both field trial analysis and using hydroponic conditions that created low iron availability similar to calcareous soils. M'sehli et al. (2014) likewise observed significant variations between two *Medicago ciliaris* lines differing in their tolerance to Fe deficiency: a considerable reduction in chlorophyll amounts was detected particularly in the sensitive line. Their results revealed that at the leaf level, photosynthetic parameters of the chlorosis-sensitive line were more affected by Fe starvation than those of the tolerant line. Bicarbonate-induced Fe deficiency caused a significant decrease in leaf photosynthetic pigment content in tolerant and sensitive varieties compared to the control but the sensitive variety was more affected than the tolerant (Mahmoudi et al., 2009).

M'sehli et al. (2014) found that Fe deprivation had a lowering effect on chlorophyll content, photosynthetic electron transport activity and chlorophyll fluorescence in chlorosis sensitive cell lines. Photosynthetic apparatus in a sensitive line was noted to be severely affected by Fe deficiency treatment (M'sehli et al., 2014). They discovered that the electron transport through the photosynthetic chain was repressed by iron deprivation especially in the line sensitive to Fe deficiency. In addition, Fe deficiency was found to have a greater effect on the functionality of the photosynthetic machinery in a chlorosis-sensitive plant than in the

tolerant. Pascal and Douce (1993) reported that the functionality of the electron transport chain at the mitochondrion and chloroplast levels was impaired as a consequence of the impact of Fe deficiency stress on photosynthetic apparatus.

The photosystems are strong sinks for Fe (Raven et al., 1999; Broadley et al., 2012): PS1 (12 atoms Fe per complex) has a higher Fe content than PSII (3 atoms per complex) and cytochrome bf complex (5 atoms per complex). PSII efficiency was down-regulated in response to Fe deficiency but the activity of PSI was more affected than PSII possibly due to more Fe atoms per PSI than PSII (Larbi et al., 2006; Pushnik and Miller, 1989). Decreases in the electron transport rate, photosynthesis and effective quantum yield reflect damages of PS II and electron transport chains in iron-deprived plants (Tewari et al., 2013). Transport of electrons through the photosynthetic chain was inhibited by the absence of iron, specifically in the sensitive genotypes (M'sehli et al., 2014). Adequate supplies of Fe to chlorotic leaves after exposure to low Fe levels increased PS1 function more strongly than that of PSII (Larbi et al., 2006; Broadley et al., 2012).

b) Root chlorophyll content

Overall, chlorophyll levels increased in the roots of control plants and IFN plant lines compared to EF lines (Figure 44). The mean root chlorophyll content ranged from 11-21 mg/ml in control plants, 3-9 mg/ml in EF lines and 3-11 mg/ml in IFN lines. A1 root chlorophyll content was twice lower than the IFN lines (A3 and A4) and four times reduced compared to the controls but equivalent to that of the A2 IFN line. For the B set of plant lines, root chlorophyll content of B2 and B9 EF lines was decreased by about 73% compared to control plants and was 25 - 44 % lower than those of IFN lines ($p<0.05$). The B5 IFN line however, had root chlorophyll content equivalent to B2 and B9. Root chlorophyll content in other B IFN plant lines ranged from 8.4 to 5.4 mg/ml and was 3 to 4-folds reduced relative to the controls. Compared to other C plant lines, the C5 inefficient line and control plants had significantly increased ($p<0.05$) root chlorophyll levels. Root chlorophyll contents of IFN lines C1-C4 were similar to those of A3 and A4 IFN lines with values ranging from 6.8 - 7.7 mg/ml. DSK and D50 controls had significantly greater (4 to 5-folds) root chlorophyll content than the D plant lines. The mean root chlorophyll content in the E set of Fe-efficient (E1-3 and E7) was about 1-2 times decreased ($p<0.05$) relative to the controls. The E5 IFN line and controls had comparable root chlorophyll amounts while the E3 Fe-efficient line had the least amount. Root chlorophyll content in the B set of IFN lines ranged from 11.3 - 6.9 mg/ml.

The effect of Fe deficiency on roots has been studied chiefly in relation to root morphological adaptive features and growth. Few references could be found on the subject of chlorophyll content in plant roots and virtually none on root chlorophyll content in response to Fe deficiency conditions *in vitro*. It was observed in the current study that the roots of mostly control plants and some IFN plants (e.g. C5 plant line) were green and of high chlorophyll content. This occurrence/phenomenon may be relevant for visual assessment of roots in relating to the identification of IDC tolerance or susceptibility at the root level. Also, root greening and or high chlorophyll content may be used as an indicator for susceptibility to Fe deficiency in root cultures.

Plant roots growing underground as heterotrophic organs mainly develop amyloplasts which are non-photosynthetic plastids. However, plant roots have been found to possess the potential to turn green when exposed to light because light signalling is necessary for chlorophyll accumulation in roots (Yoon, 2000; Kobayashi et al., 2012). Chlorophyll functions as a pigment for light energy harvesting therefore in the presence of continuous light (24 hr photoperiod), greening (chlorophyll accumulation) can take place in the roots as observed in this current study. Roots of 'Iwa' potato stock plants cultured *in vitro* were observed to become green. This suggests that the potato root organ has potential for chlorophyll formation and photosynthesis under continuous light under *in vitro* conditions. Greening of 'Iwa' potato roots during *in vitro* micropropagation was reported to be due to chloroplast synthesis (Yoon, 2000).

Protochlorophyllide, an immediate precursor of chlorophyll, was identified in the roots of seven plant species using absorbance and fluorescence spectra of acetone and ether extracts (McEwen et al., 1991). A study of the functional assembly of photosystems in roots suggests that roots can develop photosynthetically active plastids (Kobayashi et al., 2012). Transmission electron microscopy showed plastids with thylakoid membranes in the root but the membranes of these plastids were less developed than those of leaf chloroplasts (Kobayashi et al., 2012). The authors also detected that that chlorophyll biosynthesis in roots was positively regulated by cytokinin but repressed by auxin signaling.

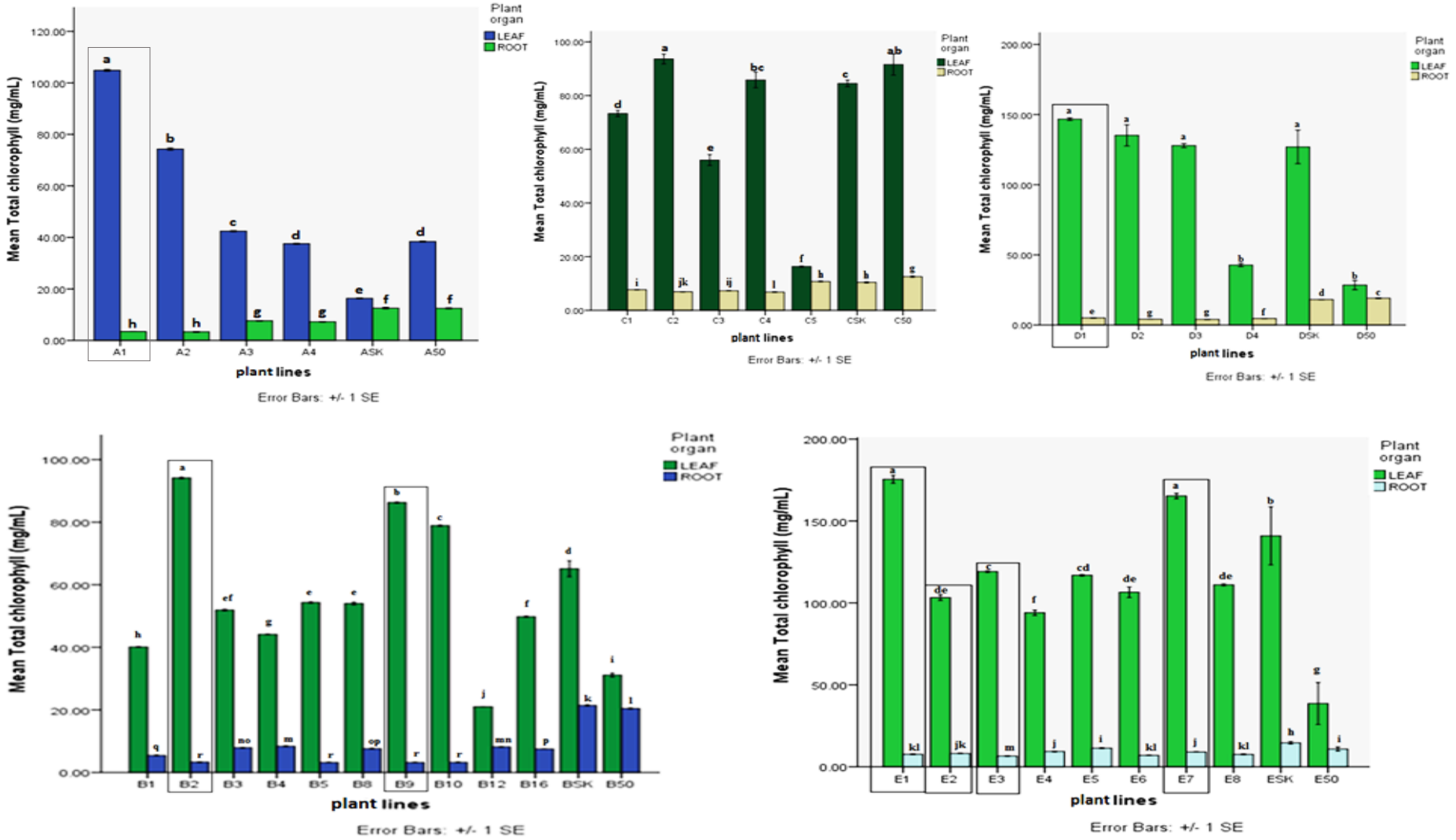


Figure 44. Chlorophyll content in young leaves of potato plant lines cultured in Fe-deficient medium for one month. Values are the means of three independent replications. Bars = standard error of the mean. Different letters indicate significant differences between means. Selected EF lines are indicated in rectangular box.

5.5.2 Carotenoid content and IDC tolerance

a) Leaf carotenoid content

There was no clear pattern in leaf carotenoid content between potato plant lines and control plants: some IDC tolerant lines had comparable, reduced or increased carotenoid contents compared to sensitive lines and control plants under limiting Fe supplies (Figure 45). Carotenoid content in the leaves of the selected tolerant plant lines (A1, B2, B9, D1, E1-3, E7) was less than 1 mg/ml except that of E1 (1.4 mg/ml) while in the controls the levels ranged from 0.5-2.5 mg/ml. Leaf carotenoid content in the A1 Fe-efficient line was 43-56% less than in control plants ($p<0.05$). Increased leaf carotenoid levels were found in control plants and 60% of the IDC sensitive lines (A3, A4) but not in A2 which, had drastically decreased (least value) carotenoid contents. Leaf carotenoid contents in 60% of the B lines was not different from that of the controls ($p<0.05$). Although the IDC tolerant lines B2 and B9 had similar leaf carotenoid levels, the amounts were about twice more than in the controls ($p<0.05$). All C lines (IDC sensitive) had reduced leaf carotenoid contents compared to controls except for C5 which had a value 2-folds greater than those of the controls. All E lines except for E1 had significantly reduced leaf carotenoid content relative to the controls. Leaf carotenoid levels measured in B2, B9, E1 and D1 potential Fe-efficient lines were higher than the parental biotype (SK). Fe-efficient and inefficient plant lines with equivalent leaf carotenoid amounts were D1 and D2, E2-3, E7 and E4, E5 and E6.

b) Root carotenoid content

In both EF and IFN plant lines, Fe deprivation induced a decrease in root carotenoid content (Figure 46). Nevertheless, Fe deficiency had a marked influence on carotenoid content principally in the EF than IFN lines. The reduction in total carotenoid concentrations observed in the EF plant lines ranged from approximately 25-72 %. Root carotenoid content in all A plant lines declined (by 1-3 times) significantly ($p<0.05$) compared to control plants under limited Fe supplies but the reduction was more pronounced (about 3-folds) in the Fe-efficient line, A1. Carotenoid contents in the roots of all B lines were significantly lower (by 2 to 6-folds) than those of the control plants. However, the EF lines, B2 and B9, were more affected with a 5-6 times decrease in carotenoid contents. The IFN line, C5 and the C50 control had the highest root carotenoid content but other IFN lines had comparatively reduced carotenoid content. Root carotenoid content was about the same for CSK, C4 and C2. The

sensitive line, C5, was severely chlorotic and contained low levels of chlorophyll but had high leaf and root carotenoid levels.

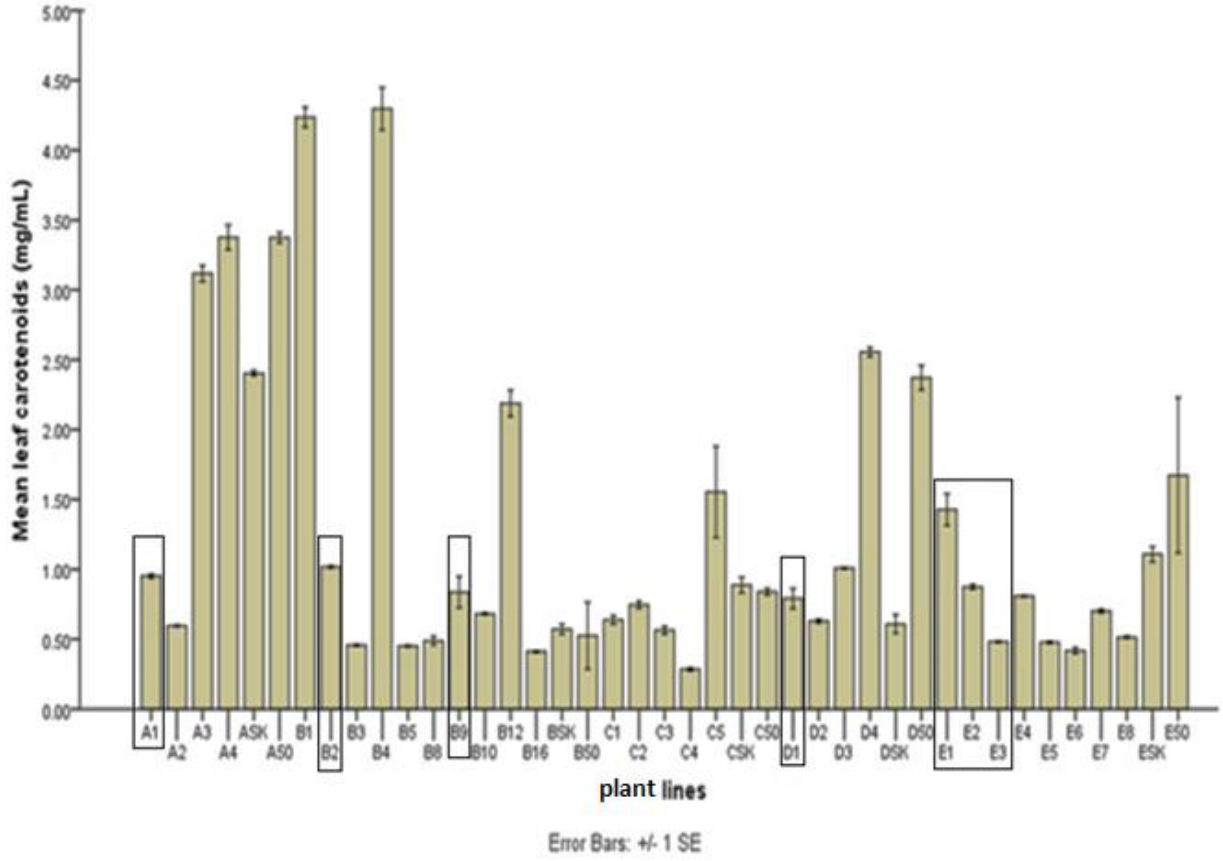


Figure 45. Leaf carotenoid content in Fe-efficient and inefficient plant lines with their controls. Potato plant lines and control plants were cultured under Fe-deficiency conditions and harvested for analysis after three months (two subcultures). Values represent the means of three independent replicates. Error bars = standard error of the means. Selected EF lines are indicated in rectangular boxes.

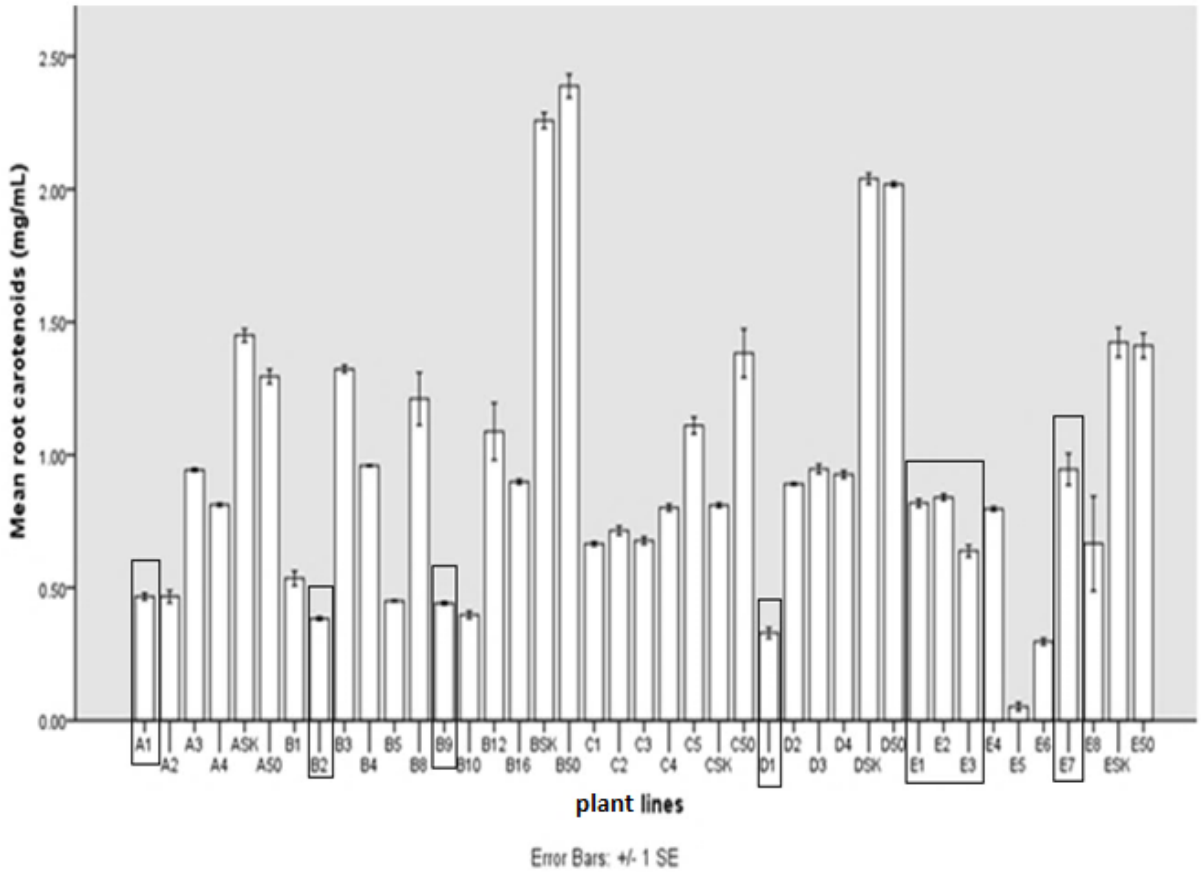


Figure 46. Root carotenoid content in Fe-efficient and inefficient plant lines with their controls. Potato plant lines and control plants were cultured under Fe-deficiency conditions and harvested for analysis after three months (two subcultures). Values represent the means of three independent replicates. Error bars represent standard error of the means. Selected EF lines are indicated in rectangular boxes.

Carotenoid plant pigments are vital components of the photosynthetic membranes in plants. They are located in the chloroplast and absorb light energy for use in photosynthesis for plant development and survival. Carotenoid content in leaves and roots of plant lines and control plants were monitored to determine whether their levels were influenced by Fe-deficiency conditions and/or tolerance to chlorosis. Overall, carotenoid content in leaves was significantly ($p<0.05$) enhanced than in the roots. There did not appear to be a clear trend about the effect of Fe deficiency on the levels of carotenoid in potato leaves and development of Fe-deficiency tolerance in plant lines. Tolerant lines had similar, reduced or increased leaf carotenoid contents compared to sensitive lines and/ or control plants under limiting Fe supplies and vice versa.

According to Mahmoudi et al. (2009), leaf carotenoid content of a chickpea chlorosis-tolerant line was barely modified by a direct Fe-deficiency treatment and a lack of a significant impact was noted for the carotenoid content in the sensitive line. Conversely bicarbonate-induced Fe deficiency caused a significant decline in leaf carotenoid content with a 62% reduction in a chlorosis-sensitive line and 42% in a tolerant line. An earlier report indicated that Fe-deficiency could cause a large decrease in plant carotenoid contents (Iturbe-Ormaetxe et al., 1995; Abadia, 1999) but carotenoid is generally less affected than chlorophyll under conditions of Fe shortage (Broadley et al., 2012). Fe deficiency decreased carotenoid contents in *B. napus* leaves (Tewari et al., 2013). Growing sugar beet plants hydroponically under a limited iron supply decreased certain carotenoid components (neoxanthin and beta-carotene) nonetheless, carotenoid within the xanthophyll cycle were less affected in leaves (Morales et al., 1990). The authors noted that the magnitude of the decrease was related to the specific pigment considered. Furthermore, they found that Fe-deficiency decreased the content of chlorophylls more than carotenoid. Fe deprivation decreased total leaf carotenoid concentration in lettuce leaves (Msilini et al., 2013) and in three other plants investigated by Larbi et al. (2006). Taking together all the results of various studies, it is suggested that genotypic and varietal differences can influence carotenoid content in plants as well as tolerance to Fe deficiency. Carotenoid may differ depending on their quantity in the plant.

Recently, Beltran et al. (2015) discovered that the regulation of carotenoid biosynthesis is through a heme-based *cis-trans* isomerase. They proposed that isomerisation of carotenoid depends on a ferrous heme b cofactor that undergoes redox-regulated ligand switching between the heme iron and alternate cis-carotene isomerase residues. However, the exact

mechanism of how limiting Fe supplies influence carotenoid synthesis appears not to have been fully explored.

There is lack of information on root carotenoid contents of plants under Fe-deficiency conditions. Based on the current study, root rather than leaf carotenoid content appears to be a more suitable indicator of IDC tolerance in potato plants exposed to conditions of Fe shortage *in vitro*. It is not clear nor well understood whether the wide diversity in leaf carotenoid content was influenced by variations in leaf chlorophyll or the Fe-deficiency treatment to which each group of plant lines (A-C) was exposed to. Carotenoid production in leaves did not appear to be affected by iron starvation, an indication that regulation of carotenoid biosynthesis in leaves may be different from that of roots under Fe deficiency conditions.

5.5.3 FCR activity associated with Fe-efficiency

To investigate the biochemical basis for the differential tolerance to IDC among Fe-efficient callus-derived potato plant lines, FCR activity was evaluated after exposure of plants to low-Fe availability. FCR activity was assessed to test the hypothesis that FCR activity levels in the root and leaf of Fe-efficient plants were elevated relative to inefficient and control plants growing under Fe-deficiency stress conditions.

a) Leaf FCR activity

Generally, leaves of potato plant lines exhibited characteristic Fe-efficiency response: elevated FCR activites when cultured on Fe-deficient medium (Figure 47). FCR activities were higher in the Fe-efficient plant lines compared to the their respective controls. Leaf FCR activity increased in plant lines with a decrease in the Fe deficiency status (from 0.005 to 5 µM) of the test medium. For example, the A1 line exhibiting IDC tolerance on 0.005 µM Fe medium ~~(A)~~ had lower FCR activities compared to the selected Fe-efficient B (B2, B9) and E lines (E1-3, E7) exposed to 0.5 and 5 µM Fe media, respectively. Interestingly, some IFN lines (D2-4, B3, E9) and controls exhibited increased leaf FCR activities compared to their counterpart EF lines (D1, B2, B9, E3).

Leaf FCR activities in all A lines were higher (by 1.5 to 1.7-folds) than those of the control plants. FCR activities were highest in lines A1 and A3. The mean differences in leaf FCR activities between the controls and all A lines except A4 were statistically significant

($p<0.05$). Interestingly, the A3 in which a higher degree of chlorosis was detected compared to the A1 Fe-efficient line showed the highest root and leaf FCR actitives. Approximately 44% of the B plant lines including B2, B9 Fe-efficient ones had leaf FCR activities significantly higher than their controls. B3 however, exhibited the higherst mean leaf FCR activities though it was classified as Fe-inefficient based on an average VCR score of 2.50 relative to 1.38 and 1.33 for B2 and B9 respectively. Mean leaf FCR activities in 56% of the B lines assayed were significantly lower than the control plants.

Compared to the controls, 40% of the C lines exhibited an elevation in leaf FCR activity while the reminder had leaf FCR activity either similar to or lower than that of the control plants. The leaves of C1 and C3 plant lines exhibited pronounced Fe-efficiency response. Leaf FCR activities in 60% of the C lines were either lower ($p<0.05$) or equivalent to the control plants. The severly chlorotic C5 line had the least leaf FCR activity.The selected EF line, D1, surprisingly showed a relatively lower leaf FCR activity compared to the Fe-inefficient D plant lines and their controls. D4 exhibited the highest FCR activity followed by D3. Nearly 91% of the E plant lines portrayed significantly elevated mean leaf FCR activity levels relative to the controls (Figure 45). These included the Fe-eficient lines, E1-E3 and E7. Although the E9 line was considered to be IFN in the original screen for IDC tolerance, it showed the highest mean leaf FCR activity alongside the EF lines, E1 and E2. Leaf FCR activity of E9 increased more than that of E1 by a small amount (0.045 µmol/gFW/h). About 45% of the E plants lines were of similar (about 1 µmol/gFW/h) leaf FCR activity levels.

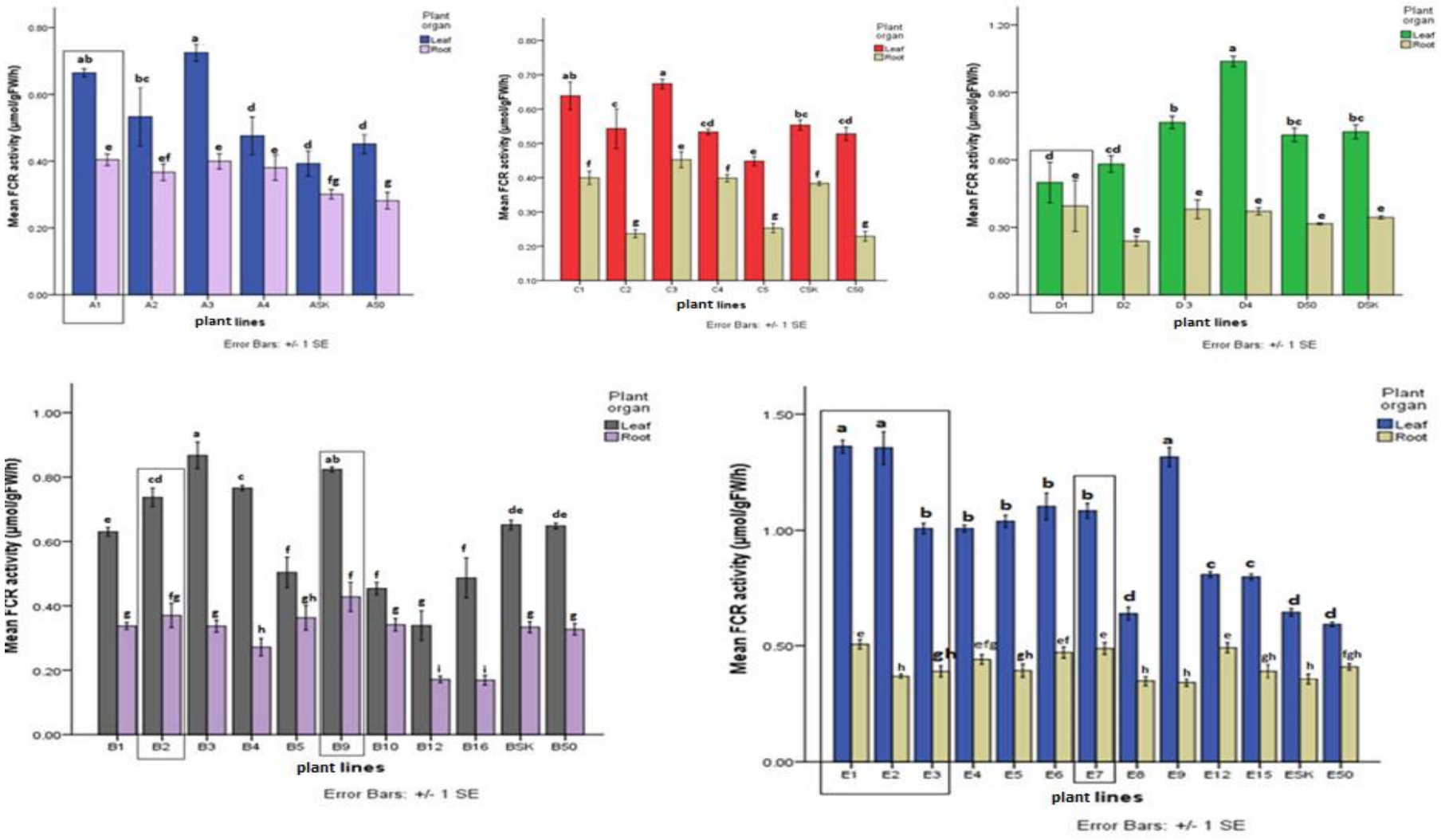


Figure 47. Root FCR activity in Fe-efficient and inefficient lines after exposure to Fe deficiency treatments. FCR was measured in intact roots harvested after two subcultures (three months) in test medium. Vertical bars ±SE indicate the means of three independent replicates and standard error of the mean. Different letters indicate significant differences between means. Selected EF lines are indicated in rectangular boxes.

Iron reduction by FCR is proposed to be an obligatory and rate-limiting step in Fe uptake in Strategy I plants (Connolly et al., 2003; Jeong and Connolly, 2009). Based on the results presented herein (Figure 47), it appears that FCR had an effect on the demand for iron uptake in EF potato plant lines compared to severely chlorotic control plants and IFN lines. This is because FCR activity was enhanced in the roots and leaves of EF plants relative to IFN and control plants. Potato plant lines exhibited the characteristic Fe-efficiency response but to a higher degree in IDC tolerant plants.

As earlier mentioned (in Section 1.2.2), FRC activity and expression has been detected at locations other than the roots indicating that the activity may not exclusively be restricted to the root. FRO (also FCR) in pea, was expressed throughout the root and leaves (Waters et al., 2002; Grotz and Guerinot, 2006) and in tomato, LeFRO1 mRNA was detected in roots and shoots (Li et al., 2004; Grotz and Guerinot, 2006). Using *in situ* hybridisation, PsFRO1 expression was identified in the mesophyll cells of pea leaves (Waters et al., 2002). Ferric chelate reductase activity was also detected in sunflower (De la Guardia and Alcantara, 1996) and cowpea leaves (Bruggemann et al., 1993). It has been suggested that FROs may be involved in Fe uptake from the soil, Fe mobilisation in the shoots and in iron distribution within plants (Jeong and Connolly, 2009; Waters et al., 2002). The results with *S. tuberosum* cv 'Iwa' clearly show that FCR activity is activated in response to low Fe supplies in intact leaves just as in roots of potato plant lines.

Leaf FCR activity in the potential EF plant lines was mostly more highly stimulated than those of IFN lines and control plants. The leaves of some IFN lines had increased FCR activity compared to their counterpart EF lines. Such IFN lines with IDC visual scores within low-medium range may be potentially Fe-efficient on the basis of FCR activity. Contrasting results were obtained using other systems rather than leaf blades excised from stem. Gonzalez-Vallejo et al. (2000) characterised FCR activity of mesophyll protoplasts isolated from Fe-sufficient and Fe-deficient sugar beet leaves and found that FCR activity decreased with Fe deficiency by approximately 65%. The authors suggested that Fe deficient leaf cells may possess intrinsic difficulties in acquiring Fe from the apoplastic space. They argued that FCR activities from leaf pieces may include other reducing activities such as the leakage of reducing compounds (organic anions) at the leaf wound. Such activities are not related to the plasma membrane of mesophyll cells (Gonza´lez-Vallejo et al., 2000). Furthermore, the authors discussed that organelles such as chloroplasts exposed to the reaction media at the wound edge may have their own FCR activity. Previous studies with leaf pieces (Bru¨ggemann et al., 1993) did not discriminate between PM-associated FCR and other

activities. In the current study, leaf FCR activity increased to a greater extent than that of roots exposed to low-Fe availability possibly due to the aforementioned reducing activities associated with the use of leaf pieces for FCR activity measurements.

It is suggested based on the current study that, leaves probably have the ability to sustain the induction of the FCR for long periods (three months on Fe-deficient medium) hence the higher FCR activity found in leaves than the roots. Additionally, leaf Fe uptake and regulatory mechanisms may be different from those of roots since higher FCR activity was observed in leaves rather than in the roots. This assumption is supported by the detection of light-regulated FCR activity in leaves of cowpea (Brüggemann et al., 1993), sunflower (de la Guardia and Alcantara, 1996) and sugar beet protoplasts (Gonzalez-Vallejo et al., 2000), but not in roots. The processes involved in the mechanism of Fe uptake by leaves are of crucial importance in understanding FCR enzyme activity in leaves. Fe is transported to the leaves probably as Fe(III) chelated by citrate (López-Millán et al., 2000) and Fe(III) is reduced in the leaf apoplast before uptake by leaf cells (Brüggemann et al., 1993). FCR activity in leaves may be required for Fe (III) reduction to Fe(II) before the reduced form can cross the plasmalemma into leaf cell (Ojeda et al., 2005). Leaf mesophyll cells contain a plasma-membrane-bound FCR which can cause the reduction of Fe-chelates in-vivo (Brüggemann et al. 1993; González-Vallejo et al., 2000). The FCR activity in the leaf plasma membrane plays a key role in iron uptake of leaf cells (Brüggemann et al., 1993). It has been demonstrated that uptake of ferric ions by leaf mesophyll depends on a reduction step as in roots (Brüggemann et al., 1993; Jeong and Connolly, 2009).

b) Root FCR activity

Root FCR activity increased in the A1 Fe-efficient line and was 15-18% significantly higher than their respective controls (Figure 47). The mean root FCR activities among the A lines were similar but higher ($p<0.05$) compared to the control plants. Mean root FCR activity was also elevated in the B2 and B9 potential Fe-efficent lines (Figure 46) and significantly different from that of the controls ($p<0.05$). While about 33% of the B lines (B16, B12, B4) showed a marked reduction ($p<0.05$) in root FCR activity, another 33% of the B set of lines exhibited mean root FCR activities that was either significantly increased (B2, B5 and B9) or similar (B1, B3 and B10) to the controls.

Root FCR activity was highest in C3 compared to other Fe-inefficient C lines (Figure 46). The mean C3 root FCR activity was almost double that of the control, C50 and 1.2 fold

more than that of CSK. FCR activities of lines C2 and C5 were equivalent to that of the C50 control but significantly decreased relative to CSK. Differences between the mean root FCR activities of 60% of the C lines and that of the controls were statistically significant ($p<0.05$). Roots of CSK control plant showed 1.7-fold higher FCR activity with 40% the C lines (C1, C4) than C2,C5 and C50. The mean root FCR avctivity of CSK control plant was 25% higher than that of C50 control.Root FCR activity in the EF line, D1, was higher than the IFN lines, D2-4 (and the control by 2-25% and 7-11% respectively. Significantly amplified FCR activity levels were found in 72% of the E plant roots compared to the contols (ESK).The highest mean root FCR activity was found in the EF lines, E1 and E7. Root FCR activity was least expressed in the contol plant, ESK , the sensitive line, E8 and tolerant, E9. Though the mean root FCR activity of E2 IDC tolerant line was slightly higher (by 0.01-0.02 µmol/gFW/h) than that of ESK, E8 and E9, the differences were not statistically significant ($p>0.05$).

FCR has been identified as responsible for Fe uptake from the rhizosphere in response to iron limitation due to its presence principally in the root hair zone of the primary and the lateral roots (Robinson et al., 1999; Waters et al., 2002; Ivanov et al., 2012). By investigating the use of root FCR enzymatic activity as a tool for screening Fe-efficient rootstock, Gogorcena et al. (2004) reported that the technique is beneficial and easier for use in commercial breeding programs aiming at obtaining Fe-efficient plant genotypes. A close connection has been observed between Fe reduction capacity of roots and Fe chlorosis tolerance where Fe-deficiency increased root FCR activity to a greater extent in chlorosis-tolerant species than that achieved in susceptible species (Gogorcena et al., 2004). Earlier studies have shown improved root FCR activity in relation to Fe-deficiency tolerance in other plants. It has been argued that soybean EF plants challenged with iron deficiency stress develop the ability to absorb and translocate large quantities of iron, contrary to INF plants (Elmstrom and Howard, 1969). An increased root ferric reductase activity and tolerance to iron-deficiency induced chlorosis were observed in soybean (Vasconcelos et al. 2006) and rootstocks (Licciardello et al., 2013). Molassiotis et al. (2006) found that in the absence of Fe, root FCR activity was stimulated in both chlorosis tolerant and sensitive rootstocks. Transgenic rice with higher tolerance to Fe-deficiency showed elevated root FCR activity and had a greater grain yield on Fe-deficient soil compared to non-transgenic rice (Ishimaru et al., 2007). Root FCR activity was only induced in IDC tolerant but not in the sensitive okra variety due to Fe deficiency (Kabir et al., 2015).

The above-mentioned findings are consistent with the results obtained in this study, and support the notion that Fe starvation induces a stronger iron reduction response in Fe-efficient than in inefficient roots. Roots of EF lines appear to have a greater reducing capacity than INF and control plants since FCR activity was generally elevated in the selected IDC tolerant plants but lowered in sensitive ones. Lower FCR activities in sensitive rootstocks were attributed to a greater requirement for Fe(III) reduction to Fe(III) to use the Fe present in the soil (Licciardello et al., 2013). As a characteristic of other Strategy I plants, it is suggested that the higher Fe-efficiency exhibited by mostly EF potato plant lines (A1, B2, B9, D1, E1, E7) could be attributable to an increase in FCR activity coupled with proton extrusion which facilitate enhanced Fe acquisition in roots. Based on the results, it is suggested that FCR activity in roots may be a key factor contributing to a plant's ability to minimise IDC aside other processes such as root hairs development, rhizosphere acidification, ethylene production (Romera and Alcántara, 2004), and organic acid release (Abadía et al., 2002).

The data on root FCR activity and IDC efficiency presented herein support the reported induction of FRO expression as a consequence of Fe-deficiency in other plants (Connolly et al. 2003; Martínez-Cuenca et al., 2013). *FRO2* gene (encoding FCR) expression increased in citrus rootstocks under conditions of Fe deprivation with the IDC-tolerant genotype exhibiting the highest expression level (Martínez-Cuenca et al., 2013). Both *fro2* expression and FCR activity were stimulated in Fe-deficient roots although this effect was more pronounced in chlorosis tolerant rootstock (Martínez-Cuenca et al., 2013). The authors noted that Fe starvation induced root Fe uptake rates and FCR activity to a greater extent in chlorosis tolerant genotype than in the sensitive one. They deduced that the determining trait for IDC tolerance among the genotypes is the capacity to enhance Fe(III) reduction in response to Fe-deficiency through enhanced FRO2 gene expression. Higher levels of FRO2 found in chlorosis tolerant plants compared to levels in chlorosis sensitive plants suggest that chlorosis tolerance could be linked to a high constitutive Fe-reducing capacity (Li et al., 2002; Martínez-Cuenca et al., 2013).

Contrary to the aforementioned findings, root FCR activity measurements in soybean cultivars of differential IDC susceptibility showed reduced FCR activity levels under conditions of Fe shortage. Root FCR activities between Fe-efficient and inefficient lines grown in Fe deficiency induced by high bicarbonate (with pH of 7.5) content were not statistically different (Vasconcelos and Grusak, 2014). Their results showed that an efficient line displayed highest FCR activity levels both at high Fe concentrations and on Fe deficient

conditions. They explained that soybean plant roots grown under a bicarbonate system may regulate the Fe reductase mechanism differently than in other culture systems. Reductase activity is vastly dependent on the cultivar type, the type of Fe chelator used and pH of growth medium (Lucena, 2000; Blair et al., 2010). Root FCR activity declines rapidly at high pH (Lucena, 2000; Vasconcelos and Grusak, 2014).

5.5.4 Fe-efficiency increased antioxidant enzyme activity

POD activity levels were measured in selected Fe-efficient and inefficient plant lines as well as control plants to ascertain the effect of Fe-deficiency on antioxidant activity in the different lines. POD activity in Fe-efficient plant lines was remarkably higher compared to inefficient ones exposed to Fe-deficiency conditions (Figure 48). Both roots and leaves showed a similar pattern of changes in POD activity in response to Fe deficiency as the Fe-efficient plant lines showed elevated POD activity compared to the controls (A50-E50). However, root POD activities were largely higher than those of leaves. Roots and leaves of control plants grown on media containing 0.005 µM Fe (ASK, A50) and 0.1 Fe (CSK, C50) were those most affected by Fe-deficiency stress since POD activity levels were significantly lower compared to controls tested on 0.5 (BSK, B50),1 (DSK, D50) and 5 µM (ESK, E50) Fe-containing media.

a) Leaf POD activity

Generally, Fe-deficiency induced elevated POD activity response in plant lines with a considerably greater effect in EF than IFN lines relative to control plants (Figure 48). The EF line, B2, had the highest leaf POD activity followed by B2>A1>B9>E1 (decreasing order) relative to the ESK control. However, leaf POD activity was markedly stimulated in the selected EF lines (A1, B2, B9, D1, E1-7) compared to the A50 to E50 controls. Lines A1 and A4 exhibited significantly increased leaf POD activities with respect to controls (ASK, A50). The A1 Fe-efficient line showed significantly elevated leaf POD activity levels compared to the IFN line, A4, and the control plants (ASK, A50). The mean leaf POD activity of A1 was 2 to 6 times higher than the controls and 1.4-folds greater than A4. Except for inefficient B5 line which exhibited significantly reduced ($p<0.05$) leaf POD activity, all other B lines asssayed for POD activity showed increased POD levels compared to the controls. The B2 and B9 plant lines were found to be IDC tolerant after culture under Fe-deficiency conditions,

had the highest leaf POD activity compared to the EF lines, D1 and E1-7. Interestingly, leaf POD activity in B5 was significantly lower than BSK contol. In the IFN C lines, C 1 and C5, leaf POD activities increased about 3 and 2-folds, respectively, compared to the C50 control ($p<0.05$).

Considering all plant lines exposed to Fe-deficiency, C1 and C5 IFN lines exhibited the least POD activity levels amongst all the lines exposed to Fe-deficiency. For the D plant lines, the highest POD activity was expressed in the leaf of the EF line, D1 ($p<0.05$). The D2 inefficient line showed leaf POD activity levels similar to the C lines. POD activity significantly increased (by 28%) in D1 relative to the IFN line, D2 and control, D50 ($p<0.05$). The mean difference in leaf POD activities between the controls and that of D1 were statistically proven ($p<0.05$). Elevated leaf POD activity levels were detected in E1-3 and E7 Fe-efficient lines compared to E50 control plants ($p<0.05$). Surprisingly, the E3 EF line had reduced antioxidant activity compared to IFN line E8 and the ESK control.

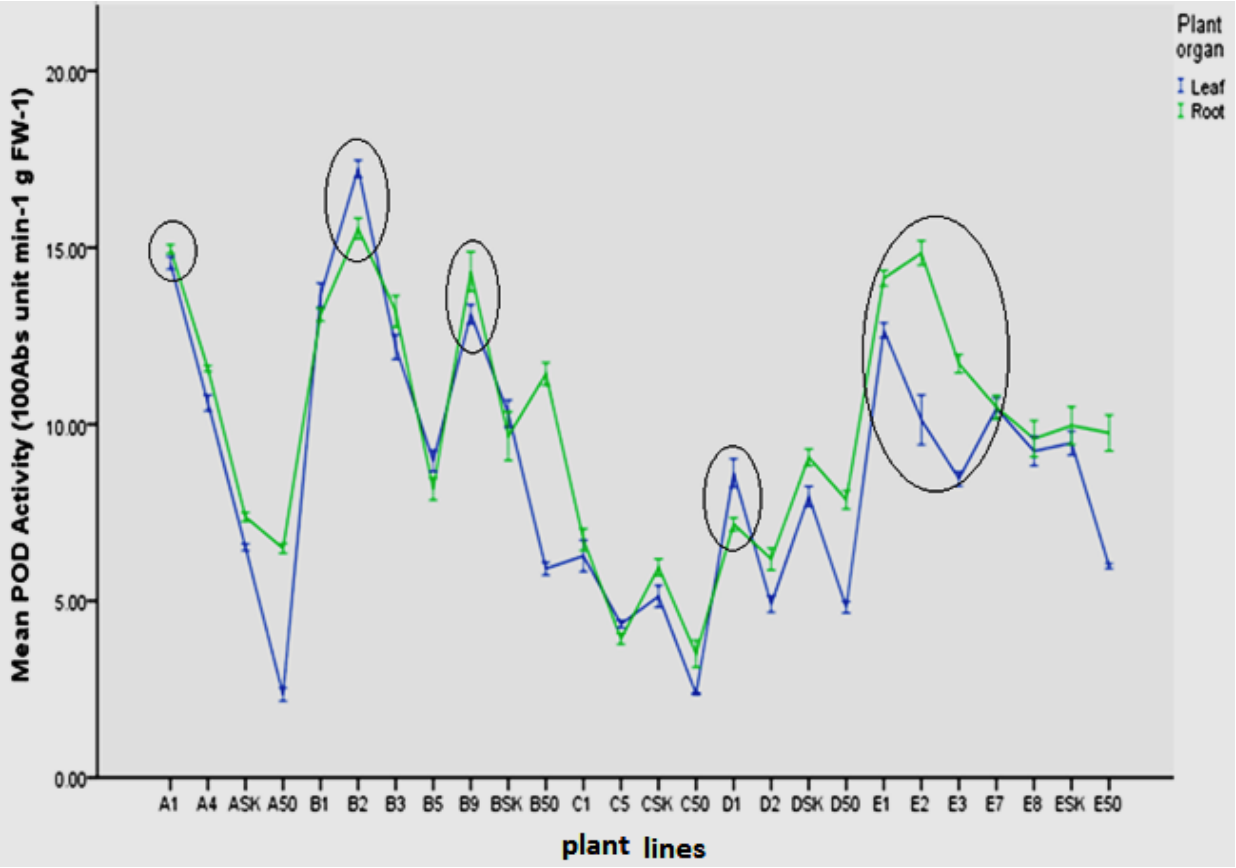


Figure 48. Enzymatic activity of guaiacol peroxidase (POD) in roots and leaves of plant lines of potato plants grown in Fe-deficient media during the treatment period of three months.

Values are the means of three independent preparations. Bars represent standard errors of the means. Selected EF lines are indicated in circles.

POD is involved in defence, lignification, ethylene biosynthesis, developmental and wound healing processes (Ranieri et al., 2001; Bhaduri and Fulekar, 2012). In the present work, the control plants having had no prior exposure to Fe-deficiency, mostly responded to the stress condition with a considerable decline in POD activity levels compared to the plant lines which were generated from Fe-efficient calli selected under Fe-deficiency condition. POD is a heme-containing enzyme and under Fe deficiency, its activity was strongly depressed in plant tissues (e.g. Ranieri et al., 2001; Tewari et al., 2013). Thus, its activity can be an indicator of the Fe nutritional status of plants. Most of the plant lines may have developed adaptive mechanisms that make and/or enable them cope under Fe-deficiency stress conditions unlike the control plants. It is assumed that the plant lines having been derived from Fe-efficient calli had probably evolved adaptive mechanisms to survive under conditions of low-Fe availability. Therefore, POD activity in calli transferred from medium with sufficient Fe supply was activated and not diminished upon reintroduction into Fe-deficiency conditions. Previous studies have noted that antioxidant defence systems differ between chlorosis tolerant and sensitive plant species or genotypes (Jelali et al., 2014; Molassiotis et al., 2006). The findings reported herein suggests that elevated antioxidant activity levels could be associated with tolerance to Fe-deficiency stress since POD activity of the chlorosis-sensitive lines were more affected by Fe starvation than that of the tolerant lines. In IDC sensitive plants, Fe depletion is possible as reflected visually by their high chlorosis scores and lower chlorophyll contents. As such, IFN lines may be incapable of activating and/or sustaining the biosynthesis of POD in response to Fe shortage. POD activity was general higher in the Fe-efficient plant lines and most plants with low-moderate chlorosis scores probably due to the plants' elevated antioxidative response to Fe-deficiency stress. Such responses could be utilised in screening techniques for selecting potato cultivars tolerant to Fe deficiency.

It has been reported that measurement of Fe-containing enzyme activities in leaves is more reliable than quantifying total Fe content for characterisation of the Fe nutritional status of plants and for assessing genotypic variations in responses to Fe deficiency (Broadley et al., 2012). Increased leaf POD activity in EF lines in relation to decreased levels in IFN lines and even more in controls (Figure 48) reported herein may signify a higher level of protection particularly against H_2O_2 build-up in the EF lines. In consonance with the results herein, higher POD activity was observed in the leaves of IDC tolerant citrus rootstocks in

comparison to sensitive ones (Licciardello et al., 2013). M'sehli et al. (2014) data relating to leaf POD activity revealed an increase in the IDC tolerant line with respect to the sensitive one in medium with no Fe. Omission of Fe from the nutrient solution in two contrasting peach rootstocks showed a significant increase in POD activity in the leaves of tolerant plants and diminished POD activity in the leaves of chlorosis-sensitive plants (Molassiotis et al., 2006). Although, under induced (bicarbonate) Fe deficiency, leaf POD activity was significantly increased in a tolerant pea cultivar than the sensitive one, no significant change in POD activity was observed in leaves of both cultivars in the absence of Fe as compared to the control (Jelali et al., 2012).

The reduced levels of POD activity in the leaves of chlorosis-sensitive citrus rootstocks grown in Fe-deficient soils resulted in immoderate ROS levels, which cause oxidative stress (Licciardello et al., 2013). In the present study, the EF lines characterised by high POD activity possibly possessed a more efficient antioxidant defence system to limit H_2O_2 production in comparison to the IFN lines. POD activity was possibly insufficient to protect leaves of IFN plant lines and control plants against Fe deficiency. According to Jelali et al. (2012) IDC tolerant pea cultivar maintained POD function under bicarbonate (low Fe) supply, possibly to counteract overproduction of H_2O_2. They indicated that the H_2O_2 scavenging mechanism was more effective in the tolerant cultivar. Lower levels of H_2O_2 accumulated in the leaves of IDC tolerant *Medicago ciliaris* plants compared to a sensitive one suggested that the former could be better protected against oxidative stress caused by Fe starvation (M'sehli et al., 2014). In the leaves, an active participation of POD antioxidant enzyme could be related to the tolerance to Fe-deficiency-induced oxidative stress.

Fe-deficiency affected POD activity in leaves to different extents to that of roots. Reduced POD activity in the leaves of Fe-efficient plant lines compared to the roots seems to suggest that the leaves had a lower capacity to protect plants against Fe-deficiency induced oxidative stress than the roots. POD activity in the leaves of tolerant plants was observed to be lower than in the roots (Licciardello et al., 2013; Jelali et al., 2012). The requirement of iron for the functioning of both leaf photosynthetic systems and POD may explain the slightly limited antioxidant protective ability of leaves in relation to roots.

b) Root POD activity

Overall, the results (Figure 48) indicate that root antioxidant enzyme (POD) activity was stimulated in all plant lines compared to their respective controls. Notably, POD activity was more enhanced in Fe-efficient than inefficient lines. The EF line, B2, had the highest root

POD activity (15.5 units) followed by others with activity range of 14.9- 7.2 units in the order B2>A1 >E2> B9> E1> E3> E7>D1. D1 had the same level of POD activity the IFN line, C1 and A controls (A50, ASK). A1 IDC tolerant line showed significantly increased root POD activity compared to the IDC sensitive line, A4, and the controls (ASK, A50). The mean POD activity in the roots of A1 was 1 and 2-folds higher in the controls and the A4 line, respectively.

Among the B lines, the IFN line, B5 had the lowest root POD activity was significantly lower ($p<0.05$) than the BSK control by one-fold. The antioxidant activity levels in B3 and B1 were similar (13.1 and 13.2 units) but significantly higher (by 7-15%) than controls and lower (by 4-8%) than the B2 and B9 EF lines. Root POD activity was more than the leaf POD activity in 40% of the B lines assayed but the reverse was observed in the other 60% (B1, B2, B5). Root POD activity in IFN line, C1 (6.7 100Abs unit min^{-1} g FW^{-1}) was higher than that of C5 (by 26%) and controls (by 6-32%). The C5 IFN line and controls (CSK, C50) recorded the lowest root POD activities (3.5-5.9 100Abs unit min^{-1} g FW^{-1}) compared to all other groups of plant lines (6.2- 15.5 100 Abs unit min^{-1} g FW^{-1}). Root antioxidant activities were mostly significantly more than leaf POD but the mean difference in leaf and root POD activies in C5 was not statistically significant.

Root POD activity of EF line, D1 was either reduced or equivalent to DSK or D50 control plants while that of IFN D2 line decreased significantly with respect to the controls. However, root POD activity levels significantly increased in D1 relative to the IFN line, D2. All the Fe-efficient E plant lines showed elevated POD activity levels compared to the controls and inefficient line. The mean differences in root POD enzyme activities between EF plant lines (E1-3, E7) and controls (E50, ESK) were statistically significant. About 5-22 % increment in root POD activity was detected in EF lines E1-3 and E7 relative to IFN line, E8. The IFN line, E8, had POD activity levels the same as the controls ($p>0.05$).

The findings of this study support the hypothesis that the POD antioxidant system is stimulated to different degrees in the two categories of plant lines (EF and IFN) explaining their different tolerance to Fe deficiency. Preceding studies have reported findings consistent with the results of this study. The exclusion of Fe from growth medium induced a significant increase in POD activity of roots of tolerant peach rootstocks while POD activity diminished in the sensitive cultivar (Molassiotis et al., 2006). The absence of Fe in the growth medium led to a decrease in POD activity in tolerant plants whereas in the susceptible, it increased but the presence of bicarbonate induced a considerable increase in the tolerant, while a reduction

was detected in the sensitive plant (Donnini et al., 2011). Roots of IDC tolerant pea cultivar showed a slight increase or similar levels of POD activities with respect to the sensitive one under conditions of Fe omission. However, the presence of bicarbonate caused a drastic decline in POD activity in the sensitive cultivar compared to the tolerant (Jelali et al., 2012, 2013). The authors put forward that POD activity might be an important contributor to pea tolerance to Fe deficiency; the adaptation of tolerant pea cultivar to bicarbonate induced Fe deficiency appear to be related to its superior ability to enhance the antioxidant response at the root level. Donnini et al. (2011) inferred that the incorporation of bicarbonate in growth medium negatively affects root POD enzymes in chlorosis susceptible plants. An additional POD isoform was detected in the roots of tolerant peach rootstock (Molassiotis et al., 2006) and pea (Jelali et al., 2013, 2014) cultivars exposed to Fe-deficiency. The authors suggest that a distinct antioxidant mechanism, adequate for protection against Fe deficiency stress might be activated in response to the Fe deprivation in the tolerant cultivars. According to Molassiotis et al. (2006), increased POD activity may be an important attribute linked to chlorosis tolerance in peach rootstocks.

The increased POD activity levels in the roots of Fe-efficient potato plant lines may be due to their greater ability to scavenge and/or limit H_2O_2 production under Fe-deficient stress relative to other IFN line and control plants. A higher level of POD in the roots of tolerant rootstocks with respect to sensitive ones was claimed to be an indication of a mechanism to prevent damage from Fe deficiency-induced oxidative stress (Licciardello et al., 2013).The detection of low H_2O_2 generation in roots of chlorosis tolerant pea, pear and quince genotypes under conditions of Fe deprivation was suggested to be as a consequence of the major increase in POD activity (Jelali et al., 2013a; Donnini et al., 2011).The high H_2O_2 build-up detected in the roots of the sensitive genotypes was ascribed to the low detoxification activity of POD due to low Fe availability.

POD is plentiful in the cell walls of the epidermis and requires phenolic compounds and H_2O_2 as substrates for biosynthesis of lignin and suberin (Broadley et al., 2012; Ranieri et al., 2001). Alterations in cell wall formation of rhizodermal cells under Fe-deficiency may be related to impaired POD activity (Broadley et al., 2012). The H_2O_2 scavenging activity of PODs primarily plays a detoxification role in the cell wall (Ranieri et al., 2003, 2001). Donnini et al. (2011) associated the detection of H_2O_2 with the modifications of mechanical properties of the cell wall during Fe deficiency stress adaptation. The presence of ROS in root apoplast of sensitive plants was correlated with lignin deposits in external layers and in endodermis of roots (Donnini et al., 2011). Peroxidases which are involved in lignin

biosynthesis may build up a physical barrier against poisoning of heavy metals stress (Rai et al., 2004; Bhaduri and Fulekar, 2012).

5.5.5 Total phenolic content and IDC tolerance

Total phenolic content was monitored to find out whether their amounts differed among EF, IFN plant lines and control plants. The results in Figure 49 shows that total phenolic content was generally higher in the leaves and roots of control plants than in the plant lines.

a) Leaf phenolic content

Overall, phenolic content in leaves of both cell line types were reduced relative to controls especially the parental biotype as presented in Figure 49. Nonetheless, 50% of the potential Fe-efficient lines (B2, E1-3) had increased phenolic contents with respect to the B50 control plant. The mean total leaf phenolic content of the control was similar but considerably higher ($p<0.05$) relative to the A plant lines. The EF line A1, recorded the highest leaf phenolic content (3.6 mg GAE/g) among the A plant lines. This value was significantly more than that of A3 (by 1.5-folds) but similar to that of IFN lines A2 and A4 (i.e. 3.4 and 3.2 mg GAE/g).

Leaf phenolic content of the B lines ranged from 2.14 (B9) to 4.80 mg GAE/g (B16). The EF line, B2, with other IFN lines (B16, B10) recorded the highest mean leaf phenolic content. The amount was equivalent to BSK and significantly greater than B50 (by 1.3-folds). The B9 EF line however, exhibited the least phenolic content with values 1.7 to 2.1-folds less than the control plant. The total leaf phenolic contents in about 70% of the B plant lines were found to be higher than those found in the B50 control.

Relative to the controls 60% of the C lines had leaf phenolic content equivalent to heir controls while 20% were of considerably lower value. IFN line, C4 had increased ($p<0.05$) leaf phenolic content than that of the controls (by 1.2-folds). Total leaf phenolic content was found to be significantly more in the controls than D4 cell line by 14%. The control plants (DSK, D50) had leaf phenolic content the same as 75 % of the D plant lines including the EF line, D1. Of all the E plant lines, leaf phenolic content was highest in the E1-3 EF lines. These Fe-efficient lines had leaf phenolic contents higher by 1.2 to1.5-folds than E50 control ($p<0.05$) but reduced (by 1.2 to1.3-folds) relative to ESK control ($p<0.05$) which had the highest leaf phenolic content. Surprisingly, the E7 Fe-efficient line had the least amount of leaf phenolics.

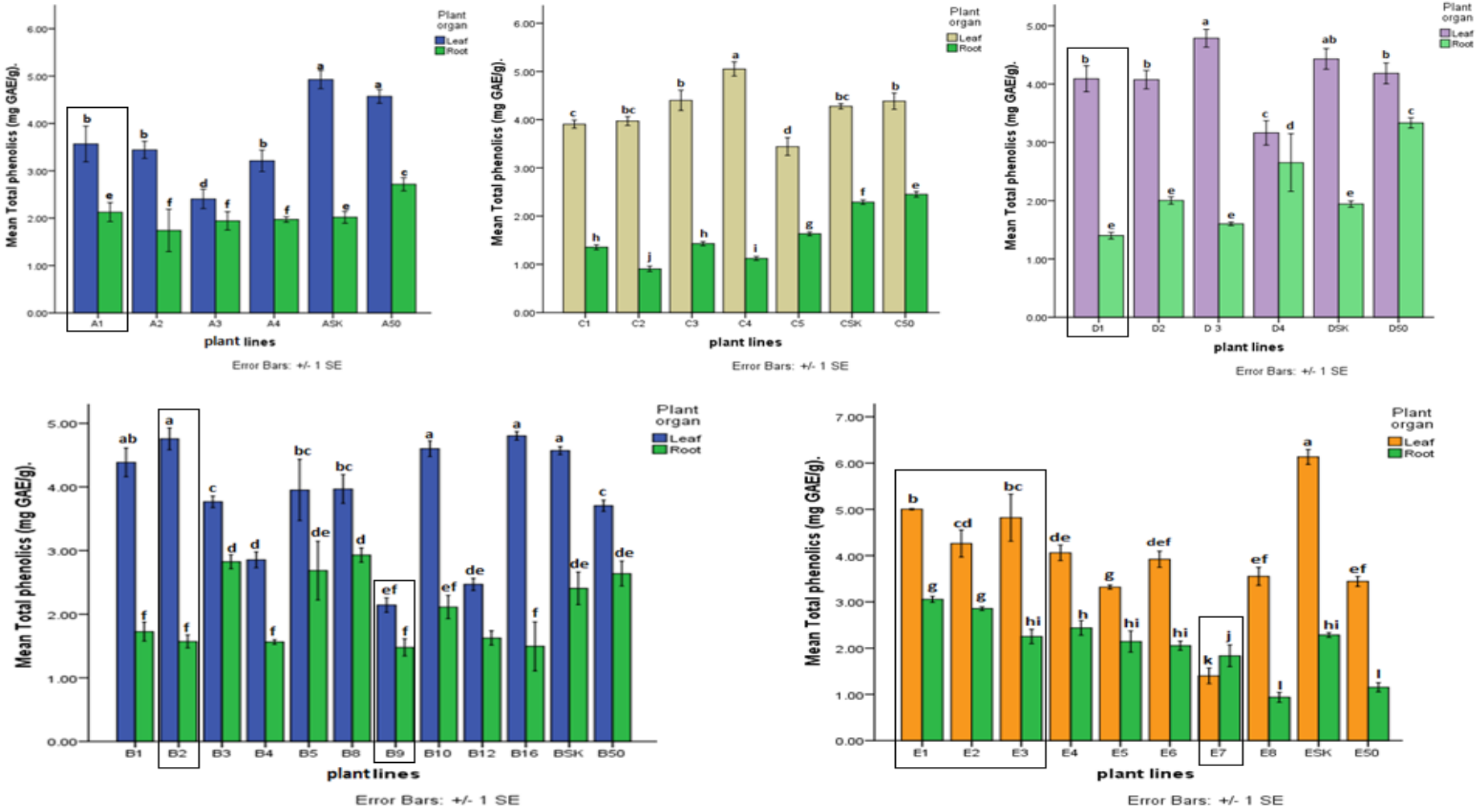


Figure 49. Effect of Fe deficiency on total phenolic concentrations in leaves and roots of potato plant lines with differential tolerance to Fe deficiency and control parental plants. Phenolic content was determined after three months' exposure to limiting Fe conditions. Selected Fe-efficient lines are highlighted by rectangular boxes. Values are means of three independent preparations. Bars= standard error of the mean. Values assigned different letters are significantly different at $p < 0.05$ according to LSD and Duncan post-hoc tests.

Phenolic compounds enhanced plant Fe nutrition by improving reutilisation of apoplastic Fe and possibly making iron more soluble (Jin et al., 2007; Rodríguez-Celma et al., 2011, 2013). Beside their chelating property, phenolics are considered to be involved in the reduction of Fe^{3+} (Jin et al., 2007; Rodríguez-Celma et al., 2011). Examination of the effect of Fe deficiency on phenolic concentration in roots and leaves in the current study revealed a reduction effect in the plant lines than controls although a few EF lines (A1, B2, E1-3) showed increased amounts in roots and/or leaves. The findings herein suggest that phenolic content may not be a good indicator of Fe-efficiency in the 'Iwa' potato plant lines.

In the present study, phenolic content in leaves was significantly higher than in roots. This finding is consistent with that of Jelali et al. (2012) who reported a higher concentration of phenolics in leaves than in roots in both IDC tolerant and sensitive cultivars. Iron deficiency decreased the amount of phenols in leaves of plant lines while an increase in the controls was recorded dissimilar to earlier findings presented below. Under limiting Fe conditions, polyphenol accumulation in leaves was found to be significantly increased in one lettuce variety, Vista, relative to another which was not significantly affected (Msilini et al., 2011). In examining the leaves of two pea cultivars, Jelali et al. (2012) found that Fe deficiency improved the activities of three enzymes of the shikimate pathway, involved in phenolic synthesis. The activities of these enzymes were higher in leaves than roots and more in the IDC tolerant pea cultivar than the sensitive one. Increase in phenolic content was greater in the leaves of the tolerant pea than the sensitive one grown in the absence of Fe (Jelali et al., 2012). Msilini et al. (2011) explained that the effective antioxidant activity of the Vista lettuce leaves after Fe deficiency, could be due to their high total polyphenolic content. Phenolic compounds can act as antioxidants by chelating metal or scavenging ROS for protection against oxidative damage (Perron and Brumaghim, 2009).

It is not clear why in the study herein, leaf and root phenolic content were mainly decreased in EF and IFN lines relative to control plants. However, some assumptions have been made in an attempt to explain this occurrence. It is suggested that due to the marked increase in POD activities especially in EF plant lines, perhaps the higher expression of peroxidases oxidised phenols to a greater extent in EF lines compared to IFN line and control plants. POD can catalyse the oxidation of phenolic substrates (e.g. guaiacol) in their detoxicant role as scavengers of H_2O_2. In IFN lines and control plants, POD activity was minimal with regards to the EF lines and as such, the phenolic dependent oxidative capacity of POD in the former reduced leading to increased phenolic contents. Non-specific peroxidases depend on phenolic compounds for the scavenging of H_2O_2 by the oxidation

of phenols (Ranieri et al., 2001). In EF lines, phenolics may have participated in the antioxidant activity of POD by serving as reducing substrates in the scavenging of H_2O_2 thus protecting the cell from oxidative injury under limiting Fe conditions.

b) Root phenolic content

The results in Figure 49 show that phenolic content in roots of potato plant lines were reduced when compared with the controls. About 40% of Fe-efficient lines (A1, E1-2) exhibited increased phenolic contents with regards to the A50 and E50 control plants respectively. In general, the A set of plant lines had mean root phenolic content either comparable or 1.4-1.6-folds lesser than the control plants (ASK, A50). The mean differences in root phenolic content among the IFN lines (A2, A3, A4) were similar ($p>0.05$) but the A1 EF line had a significantly increased in root phenolic content compared to the IFN A lines.

The mean root phenolic content was the same in 60% of the B lines but the values were significantly lower in relation to the BSK and B50 controls. Only 30% of the B lines had root phenolic content comparable to or greater than the controls ($p>0.05$). The D50 control had significantly higher root phenolic content compared to all the D plant lines screened on 1uM Fe-containing medium. Relative to the controls, the D1 EF line had significantly reduced root phenolic content. DSK root phenolics levels were 50% more than of the D plant lines. Total root phenolic content was highest in the control plants. All the C lines had 1.5 -2.7-fold reduction in root phenolic contents compared to their respective controls ($p<0.05$).

Overall, phenolic content in the roots of 87.5% and 37.5% of the E plant lines assayed were found to be increased compared to the E50 and ESK controls respectively. The Fe-efficient plant lines (E1-3, E7) had root phenolic content significantly greater (by 1.6-2.6-folds) than the E50 control. Although root phenolic contents in 50% of EF lines (E1, E2) were significantly higher (by 1.3-folds) than ESK control the others (E3, E7) had phenolics levels similar to ESK ($p>0.05$).

Research on the secretion of phenolics by red clover roots presented proof of a role of Fe deficiency-induced production of phenolics in Fe acquisition (Jin et al., 2007). In Strategy I plants, phenolic root exudates are released at a higher rate in Fe-deficiency than Fe-sufficient plants (Donnini et al., 2011; Jin et al., 2007; Jelali et al., 2012). Contrarily, it was found in the present study that total phenolic concentration mainly decreased in roots of EF and IFN plant lines compared to respective control plants under Fe deficiency conditions.

Previous studies have shown increased phenolic content in response Fe deficiency. Under Fe deficiency conditions, larger concentrations of phenols were observed in roots of chlorosis tolerant and susceptible lines of *Medicago ciliaris* than in controls (M'sehli et al., 2008). Notably, the largest increases in amounts of phenols in roots were found in the tolerant line. Likewise, Jelali et al. (2012) reported a significant increase in phenols in response to Fe-deficiency with an outstanding greater rise in the roots of a tolerant cultivar grown in the absence of Fe. Phenolic contents in roots of okra cultivars with differential tolerance to Fe deficiency were increased due to Fe deficiency compared to the plants grown on Fe sufficient hydroponic culture (Kabir et al., 2015). However, the increase was observed to be more prominent in Fe-efficient cultivar. In the study herein, Fe-efficient lines (E1-2) and a few inefficient lines (E4, B8, B3) showed an accumulation of phenolics under Fe deficiency compared to controls in agreement with the results of the aforementioned findings. According to Kabir et al. (2015) higher phenol contents in IDC tolerant Okra compared to sensitive cultivar in Fe-limiting conditions suggests that the tolerant plant was more efficient in utilising phenolic compounds to function in Fe chelation and reduction, radical scavenging and Fe reutilisation. It was also reported that Fe deficiency-induced secretion of phenolics by plant roots improved plant Fe nutrition by enhancing reutilisation of apoplastic Fe which enhanced Fe nutrition in the shoot (Jin et al., 2007). This result gives further support to the notion that Fe deficiency-induced phenolics plays a key role in Fe-efficiency in Strategy I plants like potato. Jelali et al. (2012) showed that Fe deficiency led to enhanced activities of enzymes required for phenolic biosynthesis with a greater increase in the root of a tolerant pea cultivar when compared to the susceptible one.

5.6 Test for relationships between visual chlorosis score and biochemical characterisation

This section presents results and discusses the correlation between VCR scores and the biochemical parameters assessed in the potato plants challenged with Fe-deficiency. Correlation analysis was performed to investigate-whether there was a link between visual chlorosis symptoms based on which plant lines were classified as EF or IFN and the biochemical responses exhibited by the plants when exposed to low-Fe availability. The correlation analysis was used to test the hypothesis that the differential tolerance of potato

plant lines to Fe deficiency is related specifically to their chlorotic nature and other biochemical manifestations or characteristics of their leaves and roots

a) Relationships between biochemical characteristics of plant lines at the leaf level

The results of the correlation coefficients from correlation analysis between biochemical variables and visual chlorosis score are presented in Table 12. At the leaf level, VCR score was significantly correlated with four (80%) out of five parameters assessed. Therefore, a strong correlation was found between the biochemical characteristics of the potato lines and visual chlorosis rating scores. There were significantly negative correlations between carotenoid and chlorophyll contents, FCR activity and phenolics, VCR and chlorophyll, VCR and FCR, VCR and POD activity, FCR activity and phenolics. Positive correlations occurred between VCR and carotenoid content, chlorophyll and POD activity, FCR activity and chlorophyll, phenolics and chlorophyll, POD and FCR activity.

The results support the hypothesis that there was a relationship between the visual chlorosis ratings and biochemical responses measured in leaves of plants tested under Fe-deficiency. Typically, the correlation between VCR and total chlorophyll content was significantly negative indicating that leaves of chlorosis tolerant plants of low VCR contained high amounts of chlorophyll. The depletion of chlorophyll in chlorosis sensitive plant leaves was revealed by the reduced chlorophyll contents measured in IFN lines and control plants (as reported in section 5.5.1) which was significantly related to high VCR scores ($r = -0.61$, $p<0.01$).The inverse correlation of high VCR with low POD and FCR enzyme activities as well as diminishing chlorophyll content (Table 12) in leaves suggests that in severely chlorotic leaves of IFN plant lines and control plants, POD and FCR activities were hindered or reduced. For instance, all the C plant lines were susceptible to IDC (IFN) with mean VCR score ranging from 3.40 - 4.64 (see Table 10) and had a relatively low chlorophyll (see Figure 43) content which could explain why POD activity levels in these lines were markedly low. Additionally, leaf FCR activities of the C lines were either significantly lower or the same to the control plants.

Table 12. Spearman's correlation coefficient for biochemical responses in leaves of potato plant lines with differential tolerance to Fe-deficiency.

	Total chlorophyll	Carotenoid	FCR activity	Total phenolics	POD activity	Visual chlorosis ratings
Total chlorophyll	1.0	**-.34****	**.44****	**.19***	**.35****	**-.61****
Carotenoid	**-.34****	1.0	-.08	-.12	.02	**.23***
FCR activity	**.44****	-.08	1.0	**-.15***	**.51****	**-.58****
Total phenolics	**.19***	-.12	**-.15***	1.0	0	.01
POD activity	**.35****	.02	**.51****	0	1.0	**-.59****
Visual chlorosis ratings	-.61**	**.23***	**-.58****	.01	**-.59****	1.0

**. Correlation is significant at the 0.01 level
*. Correlation is significant at the 0.05 level

Leaves that are sensitive to chlorosis (high VCR, low chlorophyll) may lack Fe required to facilitate the function and/or synthesis of the heme-containing POD enzyme. Low antioxidant enzyme activity can imply low defence against Fe-deficiency stress and therefore, increased susceptibility to chlorosis. Hence, POD activity was decreased even though plant leaves experienced stress implying that there could be overproduction of ROS in response to Fe-deficiency stress due to limited antioxidant activity. It is worthy to note that POD activity increased in the potential Fe-efficient lines compared to the inefficient lines and the control plants. This suggests that Fe-efficient plant lines were most capable of thriving under Fe-deficiency stress conditions due to elevated POD activity which works to scavenge the ROS, H_20_2, released under stress conditions in plants. Such lines with high POD activities would be able to maintain increased chlorophyll biosynthesis as shown (see Table 12) by the significantly positive relationship between POD and chlorophyll contents. Hence, there was a correlation between POD enzyme activity and higher Fe efficiency in leaves.

The significantly negative correlation between VCR and FCR activity gives an indication that increased or severe chlorotic symptoms in the leaves of plants on Fe-deficient medium was associated with decreased FCR activity. Leaves sensitive to chlorosis were probably incapable of stimulating FCR activity and as such, may be unable to sustain Fe-

uptake into cells resulting in chlorosis as evidenced by the reduced chlorophyll content in such leaves. Leaves of putative Fe-efficient lines had increased chlorophyll content with elevated FCR activities ($r = 0.44$, $p<0.01$) which supports the findings in sections 5.5.3.

Visual chlorosis symptoms were positively related to carotenoid content implying that carotenoid content increased in IFN lines with a possible greater effect in severely chlorotic leaves. This was further supported by the negative relationship detected between total chlorophyll and carotenoid content implying that when chlorophyll levels are reduced (as in IFN lines, control plants) carotenoid content are increased. Contrarily in EF lines, chlorophyll levels were increased with a decrease in carotenoid content.

Although significant, the correlation between chlorophyll and phenolic contents was weak ($r= 0.19$, $p<0.05$) which gives an indication that the few EF plant lines (e.g. B2; Figures 43 and 48) with high chlorophyll contents synthesised increased amounts of phenolics. Phenolic contents in plant lines were mainly reduced compared to the controls. Also, phenolic contents were highly variable and widely distributed among plant lines and control plants which may explain the weak relationship of phenolics with chlorosis tolerance. A similar argument can be made in the case of the low correlation between phenolic content and FCR activity ($r = -0.15$, $p<0.05$).

There was no significant linear correlation between phenolic content, POD activity and VCR in leaves of plants exposed to Fe-deficiency. Also, there was no linear correlation between POD activity and carotenoid content in leaves of plants exposed to Fe-deficiency. The lack of correlation between these parameters could also be linked to the varied trend in the phenolic and carotenoid contents among plant lines and controls in response to Fe-deficiency. Phenolics and carotenoids did not seem to be effective indicators of differential tolerance to Fe-deficiency among plant lines and the distinction between the plant lines and control plants varied (i.e. reduced, increased or equal).

b) Relationships between biochemical characteristics plant lines at the root level

The results of the correlation analysis show that chlorophyll content in the roots strongly correlated with all other biochemical parameters assessed (see Table 13). In the roots, chlorophyll content was found to be inversely related to Fe-efficiency in that plants with non-chlorotic leaves had lower chlorophyll pigments in the roots. Strong positive correlations (Table 13) were found between chlorophyll and carotenoid content, chlorophyll and phenolics, carotenoids and phenolics, FCR and POD enzyme activities. Negative correlations

were detected among chlorophyll and FCR, chlorophyll and POD, FCR and carotenoids, carotenoids and POD activity.

Under Fe-deficiency conditions, root FCR activity was negatively related to chlorophyll and carotenoid contents. This implies that root FCR activity increased with a reduction in total chlorophyll content. Roots of chlorosis tolerant plants had lower chlorophyll contents with elevated FCR indicating that Fe-efficiency at the root level was linked to decreased chlorophyll contents and elevated FCR activity. It was observed that the roots of EF lines were largely light brown or cream to pale yellow in colour and had low chlorophyll contents compared to the control plants and some IFN lines (e.g.C5) which were green and of high chlorophyll content (see Figure 44). A significantly strong relationship was found between FCR activity in roots and leaves ($r = 0.52$, $p < 0.01$); elevated FCR activity in the leaves of potential Fe-efficient lines corresponded with an increase in root FCR activity. This suggests that increased FCR activity is a good predictor of IDC tolerance in potato at both the leaf and root level.

POD activity decreased when there was an increase in root chlorophyll and carotenoid contents. However, POD activity was increased when there was a rise in FCR activity. Contrary to the leaves, roots of chlorosis tolerant lines had reduced chlorophyll contents but in the roots low chlorophyll content was associated with increased levels of POD activity (see Figures 49). This implies that there was enhanced antioxidant capacity of EF lines to limit Fe deficiency-induced oxidative stress. As discussed earlier with respect to the leaves, IFN lines and control plants may experience oxidative stress and/or damage due to sensitivity to chlorosis and decreased POD activity. It is suggested that root reductase activity is related to Fe chlorosis tolerance.

Table 13. Spearman's correlation coefficient for biochemical responses in roots of potato plant lines with differential tolerance to Fe-deficiency.

	Total chlorophyll	Carotenoid	FCR activity	Total phenolics	POD activity
Total chlorophyll	1.0	**.74****	**-.20***	**.28****	**-.33****
Carotenoid	**.74****	1.0	**-.40****	**.27****	**-.26***
FCR activity	**-.20***	**-.40****	1.0	-.01	**.49****
Total phenolics	**.28****	**.27****	-.01	1.0	.08
POD activity	**-.33****	**-.26***	**.49****	.08	1.0

**. Correlation is significant at the 0.01 level
*. Correlation is significant at the 0.05 level

Total root phenolic content was positively related to total chlorophyll and carotenoid content which may be due to the fact that chlorophyll and carotenoid content in roots were largely decreased together with reduced phenolic concentrations in most plant lines compared to control plants. Control plant roots measured considerably increased phenolic and chlorophyll contents (see Figures 49 and 44). A strong relationship ($r = 0.74$, $p<0.01$) was found between carotenoid accumulation and chlorophyll content in roots of plant lines and control plants exposed to Fe-deficiency. This is most likely associated with responses of chlorosis sensitive control plant and/or IFN lines which had higher amounts of root photosynthetic pigments. There was no linear correlation between FCR activity and root phenolic content. This result may be due to the overall inconstant and/or opposite responses of the roots of EF and IFN plant lines as well as controls to low-Fe availability. Roots of EF lines had high, low or average phenolics levels relative to IFN lines and control plants.

5.7 Test for associations between biochemical responses and exposure to Fe-deficiency

The results in Table 14 display an association between control plants or IFN plant line and the likelihood of plant leaves to produce low amounts of chlorophyll content on exposure to Fe deficiency. Specifically, a 1-unit increase in chlorosis status (inefficiency) reduced the

odds of an association with high chlorophyll contents by 0.99-folds. Similarly, being an IFN line or control plant leaf significantly reduced the odds (OR<1) of being associated with enhanced FCR and POD activities (OR<1) under Fe-deficiency stress conditions by 0.03 and 0.69-folds, respectively. At the leaf level, high carotenoid and phenolic contents were associated with control plants compared to putative EF plant lines because being a control plant significantly raised the likelihood (OR> 1) of an association with carotenoid and total phenolic (Table 14) content under Fe-deficiency conditions.

In roots exposed to Fe-deficient medium, increased FCR and POD activities significantly lowered the likelihood (OR < 1) of an association with control plants or IFN plant lines by a factor of 0.01 and 0.78 respectively. There was a strong positive association between control plant roots and total phenolic, chlorophyll and carotenoid contents. A 1-unit increment in chlorophyll, phenolic and carotenoid contents raised the likelihood (OR>1) of an association with the roots of control plant or IFN plant lines compared to EF lines.

It seems reasonable to deduce that the opposite could be true for EF lines in that, an association with high POD and FCR activity levels and increased chlorophyll content at the leaf level increases the likelihood of an association with putative EF lines. In roots, it is inferred that the Fe efficiency status of plant lines upon exposure to Fe-deficiency could be associated with high FCR and POD activity levels and low chlorophyll content.

Table 14. Odds ratio, Exp(B), values for the biochemical responses of potato plants exposed to Fe-deficiency. Control plants were tested under these conditions together with other plant lines derived from Fe-efficient callus cells.

Biochemical parameters	LEAF		ROOT	
	Exp(B)	Sig.	Exp(B)	Sig.
Total chlorophyll	0.99	0.05	4.06	0.00
Carotenoids	1.20	0.33	2.95E+03	0.00
FCR activity	0.03	0.00	0.01	0.02
Total Phenolics	2.52	0.00	2.53	0.00
POD activity	0.69	0.00	0.78	0.00

5.8 Pattern recognition in plant cell llines based on biochemical characteristics

Using PCA and CA, a pattern of plant lines with differential IDC tolerance based on their biochemical responses/characteristics can be obtained. PCA was employed as a preliminary data reduction tool to identify plant lines based on biochemical factors that explain the majority of the variance observed in a much larger number of measured variables. PCA and CA were performed to classify and/or characterise the plant lines based on similarities or differences in the biochemical variables. One output from CA is a dendrogram which is a graphical representation in which each object is arrayed on one axis and the other axis portrays the steps in the hierarchical procedure.

To ensure that the low and high measurements of the biochemical parameters assessed were treated equally, PCA requires the original variables to be normalised and be dimensionless. Varimax rotation was used to maximise the sum of the variance of the factor coefficients. The cumulative percentage of variance explained by the PCA analysis and the similarity of the pattern-recognition outputs with the results from CA were used to evaluate reliability of the results. Also, the closeness of the outputs from PCA and CA on the same dataset is a model fit indicator. PCA was also performed to classify the plant lines based on similarity or differences in the biochemical variables.

a) Grouping of plant lines at the leaf level using PCA and CA

The PCA analysis yielded two principal components (PCs) for the leaves of the various plant lines after Varimax rotation (Figure 50). In general, the PCA model explained 99.79% cumulative total variance in the data. A high cumulative percentage of variance from PCA analysis is a good model fit indicator. Communalities of all the plant lines leaf data were between 99-100%. The rotated component matrix showing factor loadings is provided in Table B5 (see Appendix B). The first principal component (PC1) contains all putative Fe-efficient plant lines (A1, B2, B9, D1, E1-3, E7) plus other Fe-inefficient lines (A2, B5, B10, C1-4, D2-4 and E4-5, E8-15). PC2 is made up solely of IFN plant lines (A3, A4, B1, B3-4, B8, B12, B16 and C5). Factor loading plot of PC1 versus PC2 shows that the E set of plant lines, 75% of the D lines and 80% of the C lines were closely linked or grouped (see Figure 50) due to similarities in their biochemical responses to Fe-deficiency. Plant lines designated as IFN were widespread between PC1 and PC2. The results show that these plant lines were distinct from the EF ones, indicating differential biochemical characteristics at the leaf level.

The PCA grouping of the plant lines into two components was comparable to the two clusters generated by CA. A dendrogram illustrating the clusters of the plant lines is shown in Figure 51. Plant lines classification using CA yielded two distinct clusters. All the potential Fe-efficient lines (A1, B2, B9, D1, E1-3 and E7) were located in Cluster 2 together with other IFN lines (A2, C1-2, C4, B10, D2-3, E4-6, E8-15). Cluster 1 consists of only the plant lines assigned INF status.

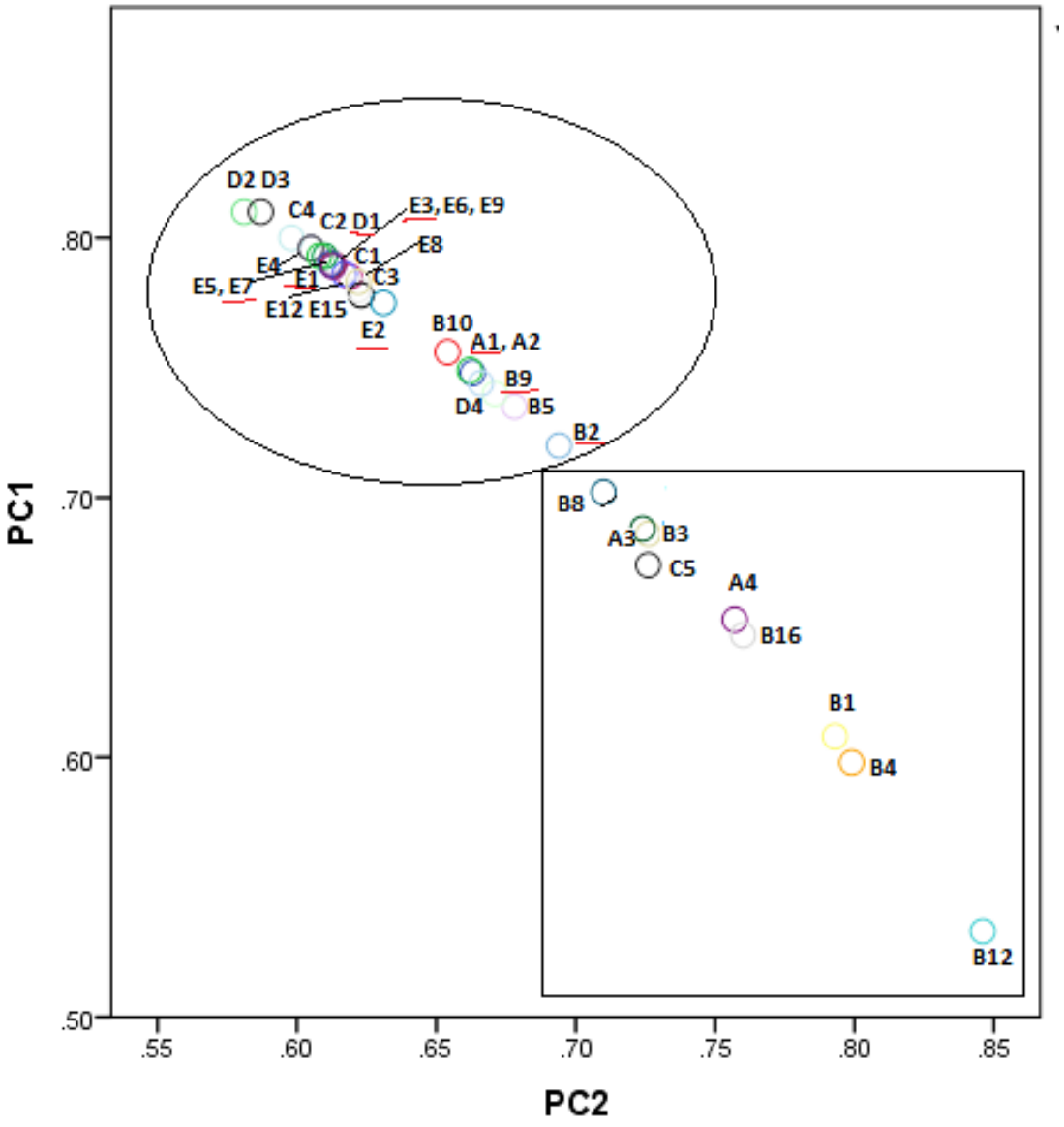


Figure 50. A plot of PC1 versus PC2 showing the groupings of potato plant lines based on biochemical responses of leaves to Fe-deficiency. Fe-efficient plant lines are underlined in red.

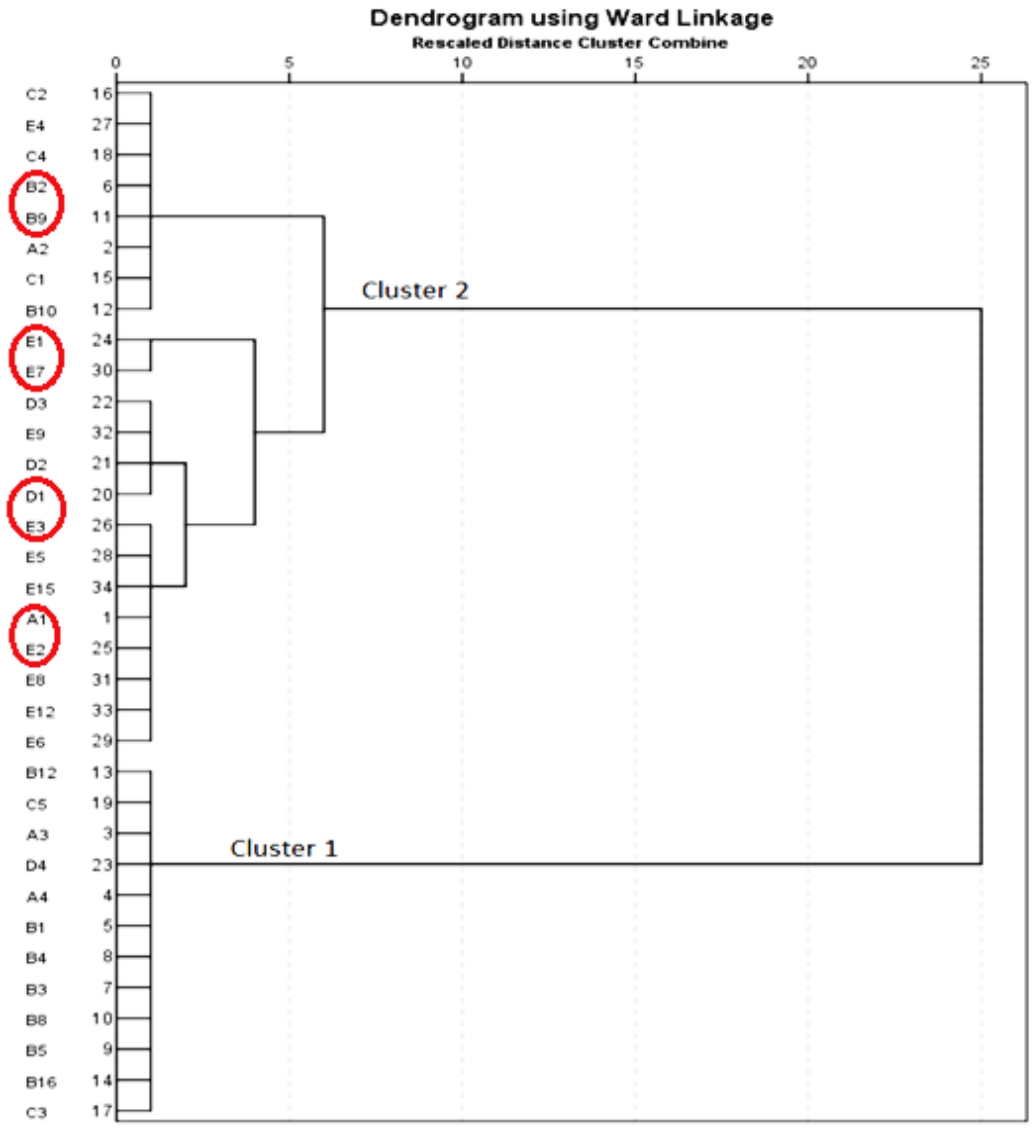


Figure 51. A dendrogram showing clusters of Fe-efficient and inefficient potato plant lines at the leaf level. Fe-efficient lines are designated in red circles.

b) Grouping of plant lines at the root level using PCA and CA

At the root level, PCA grouping of plant lines is as shown in Figure 52. The analysis produced two principal components (PC) with Eigenvalues >2.82 after Varimax rotation. The rotated component matrix showing factor loadings is provided in Appendix (Table B6). The PCA model explained 98.89% of the total variance in the data. Communalities of all the root biochemical data of the plant lines were between 94-100%. The first principal component, PC1, comprise a mixture of Fe-efficient (A1, B2, B9, D1, E1-3, E7) and inefficient plant lines (A2, A4, B1, B3, B5-8, B10, D2-4, E6) and PC2 is made up distinctively of IFN plant

lines (A3, B4, B12, B16, C1-5, E4-5, E8-9, E12-15). Overall, the plant lines within a principal component, can be interpreted as having related biochemical characteristics at the root level. All the EF lines were grouped under PC1 indicating that they have similar root biochemistry under Fe deficiency condition. At the root level, cluster analysis generated three clusters. A dendrogram illustrating the clusters of the plant lines is shown in Figure 53. Except for the EF line, E7, which belonged to cluster 2, the other seven EF lines (A1, B2, B9, D1, E1-3) were grouped in cluster 1. The majority (70%) of the members of cluster 1 were EF plant lines with only three IFN lines.

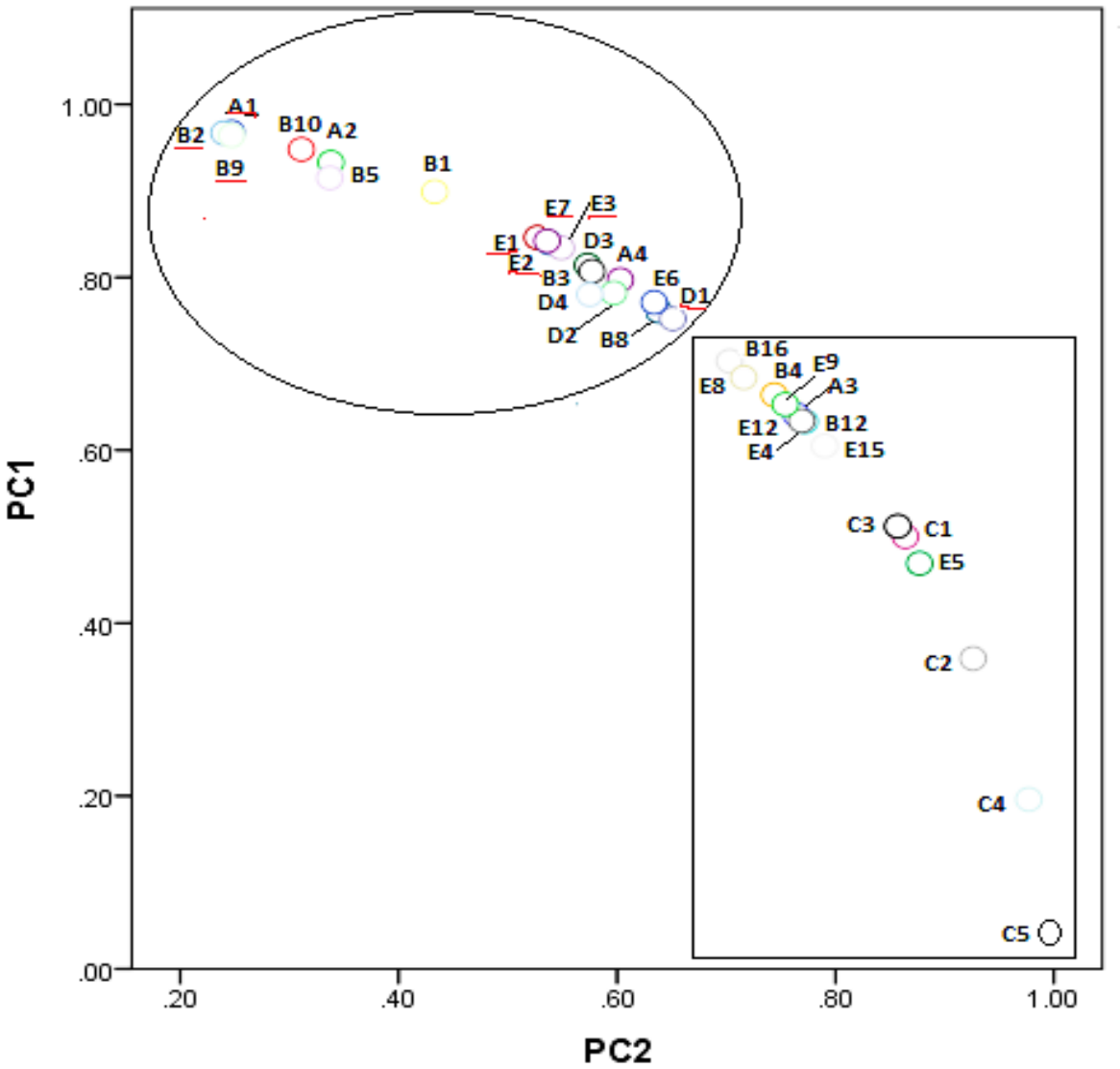


Figure 52. A plot of PC1 versus PC2 showing the groupings of the potato plant lines based on biochemical responses of roots to Fe-deficiency. Fe-efficient lines are designated in red circles.

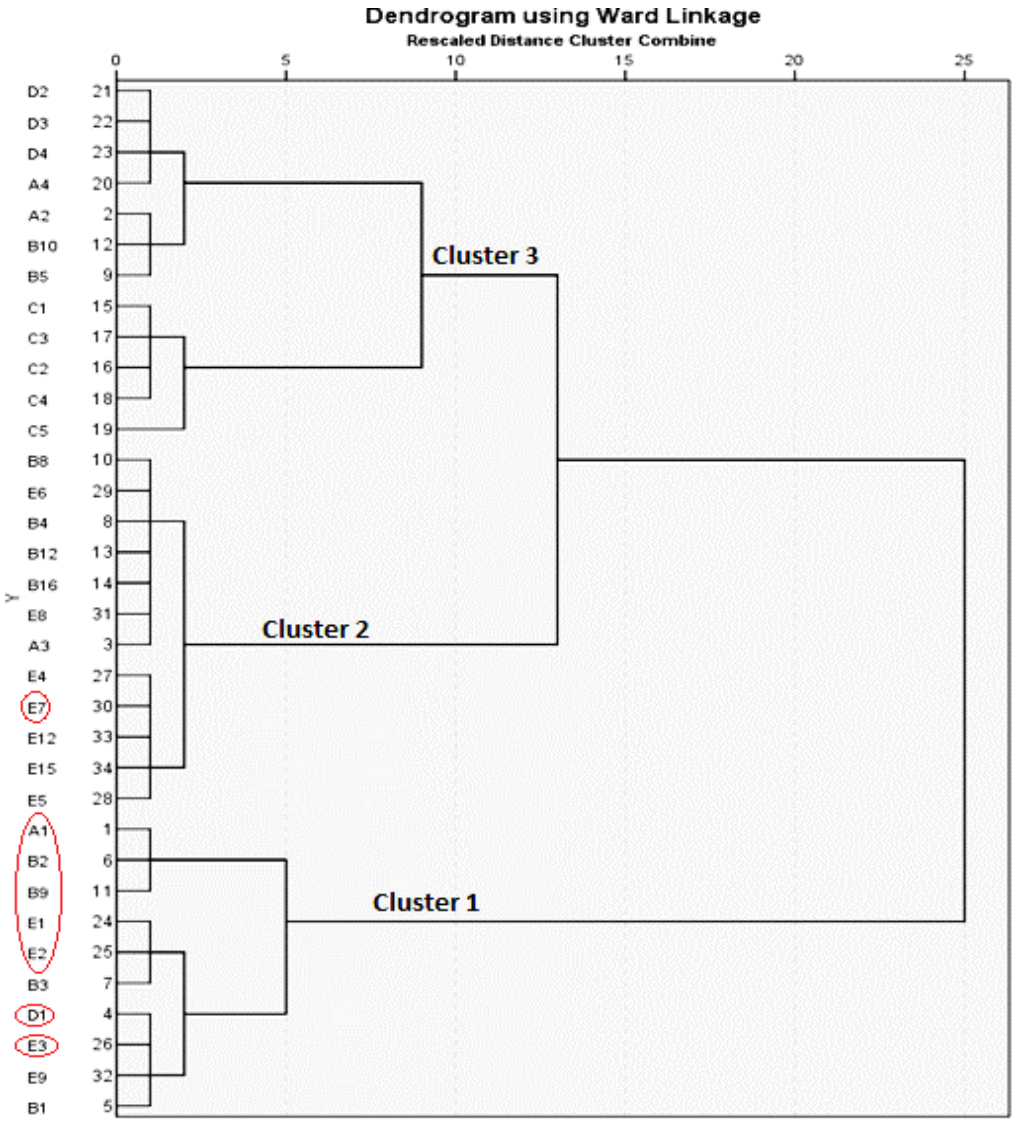


Figure 53. A dendrogram showing clusters of Fe-efficient and inefficient potato plant lines at the root level. Fe-efficient lines designated in red circles.

Plant lines within a principal component can be interpreted as having similar biochemical characteristics. At the leaf and root levels, PC1 is made up of lines (in circle) which have significantly different biochemical characteristics from PC2 (square box). PCA analysis of both leaf and root biochemical data revealed that the selected Fe-efficient plant lines were located in a common component and mostly aggregated together which may be an indication of close associations in their biochemical attributes. IFN plant lines were widely distributed among principal components both leaf and root organs. It is unclear why the PC1 collection contains the putative Fe-efficient lines and some inefficient ones but it is reasonable to assume that the lines in PC1 may have some common attributes which may not

have been noticeably evident at the morphological and visual chlorosis screening stage of the plant lines selection process. For instance, the plant lines E5, E6, E8, E9 and E15 had considerably low visual chlorosis scores (1.20 to 1.60) but were not selected as chlorosis tolerant lines because under Fe-deficiency conditions, they developed defective morphology such as very slender-thin stems, no or tiny leaves and/or had low multiplication rates. The grouping of E5, E6, E8, E9 lines (assigned IFN status) in PC1 with the selected Fe-efficient plant lines may be because they possessed Fe-efficiency potential based on their root and leaf biochemical characteristics. The cumulative differences in the biochemical parameters assayed for in the leaves and roots may not have been significantly dissimilar among the lines grouped under PC1.

The PCA results suggest that the IFN lines assembled together with the EF lines may have similar Fe-use efficiency qualities based on the leaf biochemical characteristics used for the grouping. Disparities in the source (callus) of the regenerants used to establish the plant lines and the different levels of Fe-deficiency stress conditions in which the lines were tested may have played a role in the differences in the biochemical responses and /or attributes of the plant lines and thus contributed to the PCA classifications (PC1 and PC2). In both leaves and roots, PC2 biochemical characteristics of plant lines are markedly different relative to PC1 and are heavily impacted by the various biochemical responses to Fe-deficiency. It is important to note that lines belonging to PC2 are largely chlorotic, have relatively low chlorophyll contents, low POD and FCR enzyme activities and low or high phenolic content. Classifications of both root and leaf biochemical responses to Fe-deficiency in the EF and IFN potato plant lines (Figures 50 and 52) show that line A1 share close relationship with B2 and B9 likewise D1 with E1-3 and E7.

The CA results show that leaf and root biochemistry can provide useful classifications and pattern recognition for the plant lines. The CA results indicate that plant lines belonging to a particular cluster share identical features. Plant lines within a group can be interpreted as having similar biochemical properties in response to Fe-deficiency. Using pattern recognition tools in the analysis of root and leaf biochemical indicates that membership of a particular component and/or cluster may signify Fe deficiency tolerance.

5.9 Summary

The usefulness of *in vitro* culture as an effective technique for the screening and selection of Fe-efficient plant lines in potato has been demonstrated. The aim of this study was to determine and compare the efficiency of Fe use among potato plant lines. Based on the post regeneration Fe deficiency tests, 8 (23%) Fe-efficient (A1, B2, B9, D1, E1-E3 and E7) and 27 (77%) Fe-inefficient plant lines were identified The~~se~~ INF lines developed interveinal.

Morphological characterisation of the potato plant lines revealed that the experimental plant lines had longer leaf length and more leaves per plant compared to the control parental plant. The control plants were characterised by tall bending stems (mostly slender or thin), greater internodal distance, small to medium-sized leaves and high sensitivity to chlorosis. Plant root length could contribute to IDC susceptibility in control plants and IFN plant lines since there was a significantly positive correlation between chlorosis score and root length. Stem height had a strong positive relationship with intermodal distance, leaf and root lengths.

Biochemical characteristics of plant lines were found to be largely linked to their differential tolerance to Fe-deficiency. Plant lines exhibited the characteristic Fe-efficiency response but to a higher degree in IDC tolerant cells. In terms of photosynthetic pigments, increase in chlorophyll concentration and a decrease in chlorosis symptoms were connected with Fe-efficiency at the leaf level. Chlorophyll levels in chlorosis-susceptible control plants and IFN lines were largely low under Fe-limiting conditions. Greening and high chlorophyll content in roots may be used as an indicator of susceptibility to Fe deficiency (Fe-inefficient lines). Tolerant plant lines had comparable, reduced or increased leaf carotenoid content compared to sensitive lines and/ or control plants under limiting Fe supplies and vice versa. Fe deficiency had a marked reduction effect on carotenoid content in principally EF than IFN lines. Root rather than leaf carotenoid content appears to be a more suitable indicator of IDC tolerance in potato plants exposed to conditions of Fe shortage. The results indicate that both roots and leaves of Fe-efficient plant lines repond to Fe-deficiency through the elevation of FCR activity. The findings also suggest that elevated antioxidant activity levels could be associated with tolerance to Fe-deficiency stress since POD activity of the chlorosis-sensitive lines were more affected by Fe starvation than that of the tolerant lines. The study shows that Fe-deficiency stimulated POD activity in leaves to a greater extent than in roots. Examination of the effect of Fe deficiency on phenolic concentration in roots and leaves revealed a reduction effect in EF and IFN plant lines than control plants although a few EF lines (A1, B2, E1-3) showed increased amounts in roots and/or leaves.

Test for relationships and associations between the biochemical characteristics of the plant lines showed correlations between Fe-efficiency and biochemical responses induced under low-Fe availability. Traits that are highly correlated with chlorosis can influence a plant's ability to survive in iron limiting soils. The correlation analyses suggest that *S. tuberosum* cv 'Iwa' plants having low visual chlorosis score in conjunction with higher POD and FCR enzyme activities, increased chlorophyll and phenolic contents and reduced carotenoid content in the leaves may exhibit improvement in IDC tolerance. At the root level, decreased chlorophyll, carotenoid and phenolic contents with elevated POD and FCR activities could enhance tolerance to Fe deficiency in 'Iwa' potato. It can be inferred (based on odds ratio values) that in the putative EF lines, an association with high leaf POD and FCR activity levels and increased chlorophyll content would increase the likelihood of an association with a potential Fe-efficient plant line. In roots, Fe-efficiency status of plant lines exposed to Fe-deficiency was most likely associated with enhanced FCR and POD activities and low chlorophyll contents.

Considering that the parental biotype displayed characteristic response of Strategy 1 plants (induction of FCR activity, enhanced lateral roots and root hairs formation) in the acquisition of Fe under conditions of limited Fe supplies and exhibited delayed susceptibility chlorosis, it is proposed that the parental Iwa potato plants could have inherent genetic variability for the Fe-efficiency trait. It is implied that the 'Iwa' potato plant may have a natural capacity to withstand and/or resist short term Fe-shortage or possibly have a highly effective iron homeostasis mechanism. The above-mentioned characteristics may explain why in some instances, the parental biotype (SK) control showed biochemical characteristics or responses similar to those of the plant lines generated from calli.

CHAPTER SIX

Putative Fe-efficient plant lines presented enhanced transcriptional profile of iron homeostasis-related genes

6.0 Introduction

Maintaining Fe homeostasis under control is principally critical for plant survival due to the essential role of Fe in several cellular functions. Genes play a vital role in the molecular responses to Fe deficiency stress tolerance in plants since gene expressions can be altered under such conditions (Ivanov et al., 2012; Legay et al., 2012). Iron-associated genes (e.g. *fro, irt1, fer, Nramp, vit1, fdr3)* have been identified and quantified in potato and other plants *(*Legay et al., 2012; Darbani et al., 2013; Li et al., 2004). Significant progress has been made in deciphering key players of the transcriptional networks that control Fe homeostasis and in understanding the genetic regulation of Fe uptake mechanisms in plants (Ivanov et al., 2012; Briat et al., 2007; Vert et al., 2003). Ivanov et al. (2012) reported that changes occur in the root transcriptome in response to iron deprivation: five and thirteen genes were up-regulated in roots and leaves respectively. Additionally, the transcription of genes involved in iron acquisition has been found to be upregulated in response to Fe deficiency (Kobayashi et al., 2012; Hindt and Guerinot, 2012). Genes transcriptionally regulated under conditions of low-Fe bioavailability synchronise iron uptake, translocation and storage in plant cells (Reyt et al., 2015; Legay et al., 2012). By identifying genes linked to IDC tolerance, molecular mechanisms governing Fe-deficiency stress response can be further understood. Molecular approaches are useful for identification of IDC tolerance and can aid in the development of breeding and/or transgenic strategies that can produce Fe-efficient and/or Fe-improved cultivars.

Quantitative RT-PCR (RT-qPCR), also referred to as real-time RT-PCR, is a highly specific and sensitive means of quantifying gene expression with good reproducibility and a wide quantification range (Gachon et al., 2004; Gutierrez et al., 2008; Fleige et al., 2006). It is a powerful technique for discriminating between the expression of closely linked genes and for measuring mRNA levels of weakly expressed genes (Czechowski et al., 2004; Fleige et al., 2006). To obtain accurate RT-qPCR expression measures, variations in RNA recovery and integrity, sample amount, cDNA synthesis, and differences in transcriptional activity of the tissues analysed must be controlled (Andersen et al., 2004). An essential and obligatory feature of RT-qPCR technique is the use of reference genes for appropriate normalisation in

order to improve the reliability of results (Gutierrez et al., 2008; Bustin et al., 2005) and reduce tissue-derived effects (Fleige et al., 2006).

In this chapter, gene expression responses relating to Fe transport and storage are studied in potato plant lines with differential tolerance to Fe deficiency. The expressions of two key iron homeostasis-related genes, *irt1* and *fer3*, are assessed using RT-qPCR to detect and quantify transcript levels. *IRT1 and FER3* were proposed to be involved in iron transport, storage and protection against oxidative stress (see details in sections 1.2 and 1.4). It was hypothesised that the transcriptional characteristics of EF and IFN potato plant lines were dissimilar in relation to *irt1* and *fer3* expression levels. Additionally, it was assumed that at the gene level, roots and leaves of the potato plant lines exhibited differential response to the iron deficiency.

6.1 Methods

6.1.1 Sample preparation and RNA extraction

Leaf and root samples harvested from potato plantlets were immediately frozen in liquid nitrogen and stored at -80°C. Plant material was harvested from two biological replicates each consisting of a pool of eight plantlets exposed to the same Fe-deficiency treatment *in vitro*. The samples were ground in liquid nitrogen into powder. Plant organs and equipment were maintained at low temperatures during sample homogenisation process to prevent RNA degradation. RNA extraction was carried out as outlined in section 2.7.1 and stored at -80°C before analysis. On column treatment of samples with RNase-free DNase was performed to remove genomic DNA since gDNA template could generate a false positive result and affect gene expression data. The quality of RNA was assessed using NanoDrop ND1000 spectrophotometer (section 2.7.2). The integrity of isolated RNA was evaluated by running 1% agarose gel electrophoresis (section 2.7.2).

6.1.2 RT-qPCR

High quality RNA was reverse transcribed into cDNA (see section 2.7.1). For every sample, two separate reverse transcription assays were carried out, and the resulting cDNAs were pooled to minimise error caused by variation in reverse transcription efficiency. The quality and yield of the cDNAs synthesised were assessed by performing qPCR (see section 2.7.4) on cDNA from each sample using primers to two reference genes (*L2 and EF1)* to evaluate the amplification curve and melting point curve. The qPCR reactions were carried out in

triplicates. A decreasing four-fold dilution (10-, 100-, 1000- and 10 000-fold) of cDNA pooled from all samples was used to determine the amplification efficiency of each target and reference gene (see section 2.7.5). The assay was validated by optimising primers to determine the ideal annealing temperature of all primers using 58, 60 and 62 °C annealing temperatures. Identical replicates were used to monitor the accuracy of template and reagent pipetting, homogeneity of template and instrument performance. It also allowed for obtaining results when individual qPCR reactions failed. A minus reverse transcriptase (-RT) and a no cDNA template control (NTC) were included in every RT-qPCR run to test for gDNA contamination and to detect for primer-dimers.

6.1.3 Experimental design for gene expression analysis

The expression studies were done based on the MIQE guidelines for RT-qPCR analysis (Bustin et al., 2009; Huggett et al., 2013). RT-qPCR was performed to study the relative expression of *irt1* and *fer3* genes in leaf and root organs of potato at Fe-deficiency conditions with two experimental plant lines (Fe-efficient and inefficient) and control groups (parental stock plant: conSK, plant regenerated on control medium: con50). To ensure that RT-qPCR gene expression measurements were comparable, reactions contained the same amount of RNA/cDNA and the same reaction conditions were used in all experiments (Udvardi et al., 2008). In order to minimise run-to-run variation between samples, as many samples as possible were analysed in the same run. Technical variation of measurements between samples for a particular gene was avoided, minimised or corrected. The sample maximisation strategy whereby all or as many samples as possible were analysed for a given gene in the same run was followed. This method prevents induced technical (run-to-run) variation between samples. Since all samples could not be analysed in the same run, an inter-run calibration (IRC) representing identical samples tested in every run was performed (Hellemans et al., 2007). The IRCs served as positive template controls (cDNAs pooled from all samples) and were generally included to check for consistency of reaction.

All experiments were designed with a combination of biological and identical replicates. The experiment was conducted in a 72-well plate, with each run containing two genes with two organ samples (root and leaf) of a biological replicate and IRC samples. The same pool of calibrator cDNAs was used throughout the study for each qPCR run in order to achieve consistent qPCR results. For accurate data analysis and meaningful statistics, the appropriate negative controls were included with each qPCR assay. Negative controls (-RT

and NTC) provided a means to control for contamination or other factors that may result in a non-specific rise in the fluorescence signal. Two identical and two technical replicate assays were carried out for each sample per biological replicate.

6.1.4 Data acquisition

Expression levels were defined as the number of cycles (Cq) required for the amplification to reach a threshold fixed in the exponential phase of PCR reaction. Relative quantification of *fer3* and *irt1* transcript levels was calculated taking reference genes into account based on the calculation, ΔCq Expression = $2^{-\Delta Cq}$, and the method described by of Song et al. (2012). Two reference genes (*EF1, L2*) were used to correct the relative expression of the target gene and to improve the reliability of the expression. The expression of the two reference genes was adjusted by calculating a correction factor (CF) for individual cDNAs for each reference gene using the Cq values. The final CF value was calculated by averaging the CF values (four replicates per cDNA sample) of the two reference genes and its correlation to the target genes *fer3* and *irt1* was evaluated. The Cq values of each target gene was corrected before data analysis.

Normalised relative expression data are means of relative mRNA levels in fold changes detected using four replicates for each biological replicate (n=8). Significant differences between samples were calculated using one-way ANOVA, followed by Least-significant difference (LSD) and Duncan's multiple range tests after checking the data normality and homogeneity of variances at 0.05 significance level. T-test was performed to evaluate statistical differences in mean expression levels between root and leaf organs.

6.2 Results and discussion

6.2.1 RT-qPCR optimisation and quality control

6.2.1.1 RNA quality

The quality of RNA is of foremost importance to ensure reliability and reproducibility of RT-qPCR (Fleige et al., 2006). RNA quality control analysis was performed before RT- qPCR assays to ensure that small differences in gene expression were accurately quantified. Total RNA extracts from leaf and root samples were generally of high quality. According to Fleige and Pfaffl (2006) intact RNA should be undegraded, free of nucleases, protein, genomic

DNA and enzymatic inhibitors for RT and PCR reaction. Samples with a A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.8-2.2 in TE buffer were chosen as high quality, intact RNA and used for RT-qPCR analysis. The integrity of RNA extracted was assessed by gel electrophoresis. Good quality RNA which showed two major bands of 18S and 28S ribosomal RNA which were selected for cDNA synthesis (Figure 54). A 2:1 ratio of 28S rRNA to 18S rRNA indicated that RNA was pure and of high integrity. The on column DNase treatment of RNA samples removed contaminating gDNA and DNase contamination. The purity and integrity of RNA are recognised to be critical elements for the success of RT-qPCR analysis since intact RNA is the best indication of the natural state of the transcriptome (Nolan et al., 2006; Fleige et al., 2006). The RNAs with A_{260}/A_{280} ratio less than 1.8, contaminants and/ or with high degradation were discarded and RNA extraction was repeated for these samples.

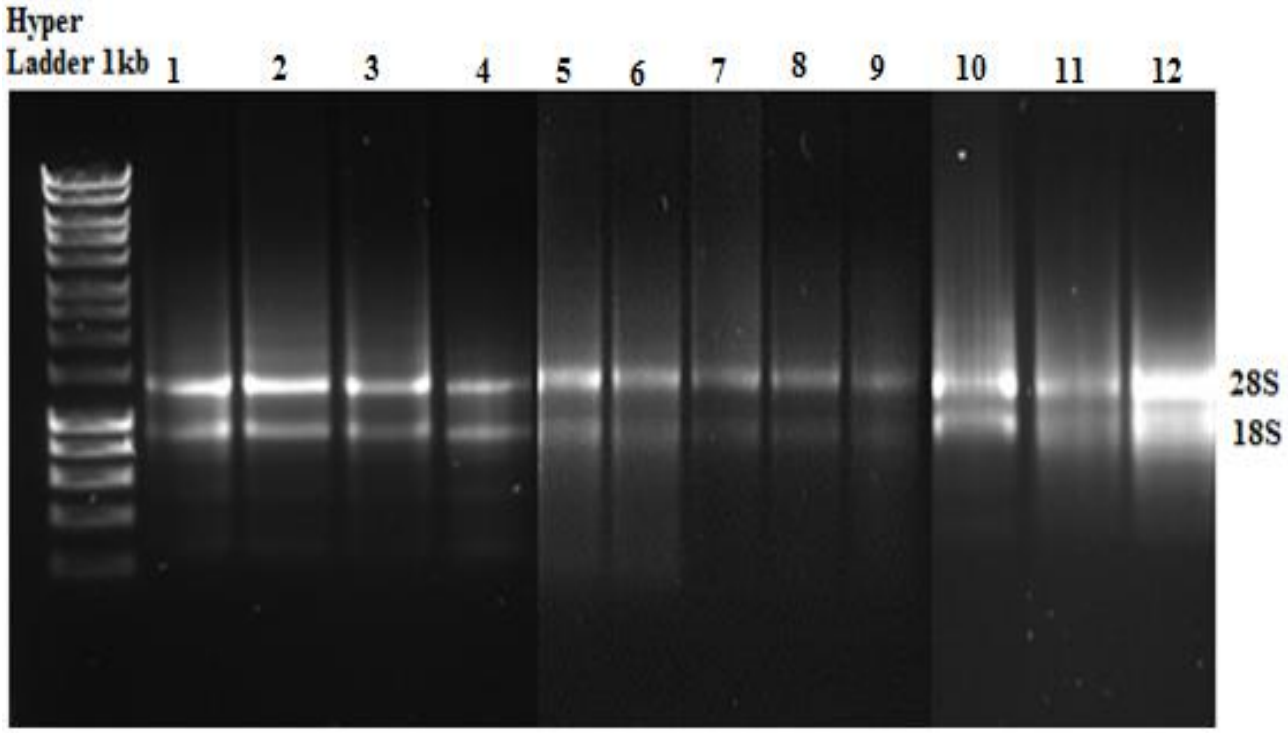


Figure 54. Intact RNA isolated using RNeasy Plant Mini Kit (Qiagen, Germany). Two µl of total RNA were separated alongside the HyperLadder 1Kb marker on a 1% denaturing agarose gel. The 28S and 18S ribosomal RNA bands are clearly visible in the leaf of B plant lines and control plant (lanes 1-4: B2, B5, B9, BSK) and in the root of A and E plant lines and control plants (lanes 5-12: A1, A2, ASK, A50, E4, ESK, E50) RNA samples.

6.2.1.2 cDNA yield and quality test

The yield and quality of cDNA was tested with primers of two reference genes that are known to be stably expressed in potato organs under Fe-deficiency and other abiotic stress conditions (Legay et al., 2012; Nicot et al., 2005). RT-qPCR reactions were performed in triplicates to assess reproducibility of the results obtained. High quality cDNAs were chosen based on the amplification and melting curve analysis of qPCR using reference genes. For example, the qPCR performed with leaf and root organs using cDNAs of control plants (Figure 55) with primers for two reference genes, *L2* and *EF1* and the target gene, *irt1*, generated distinct single peaks with specific products. The melting curve analysis of the cDNAs from leaf and root samples shows that qPCR assays produced single, specific products (Figure 55B). The amplification curve (Figure 55A) shows that all the cDNAs tested had comparable Cq values (equivalent concentrations) depending on the gene indicating that similar amount of RNAs and cDNAs and the same reaction conditions were used in all RT-qPCR reactions. Mean Cq values within the range ±1 for each reference gene across all samples indicated that cDNA yield in the samples were similar. High quality cDNAs with relatively constant Cq values for reference genes were selected for the gene expression studies.

The results demonstrate that while the target's expression levels varied, expression of the reference genes remained fairly constant. Endogenous control Cqs were lower than target Cqs. Aside treating RNA samples with DNase to eliminate possible contaminating genomic DNA, no-RT controls were used to reveal the presence of possible contaminating gDNA. A NTC (water + qPCR mix) incorporated in qPCR runs was essential to check for non-specific signal arising from primer dimers or template contamination. Ideally, no signal amplification was detected in negative control samples and the Cqs of the negative controls were at least five cycles to preferably ten cycles higher (30 to 35 cycles) than the Cqs of the least concentrated samples. PCR products amplified between 30 and 35 cycles were excluded from further analysis because they were either of too low concentration or were non-specific products.

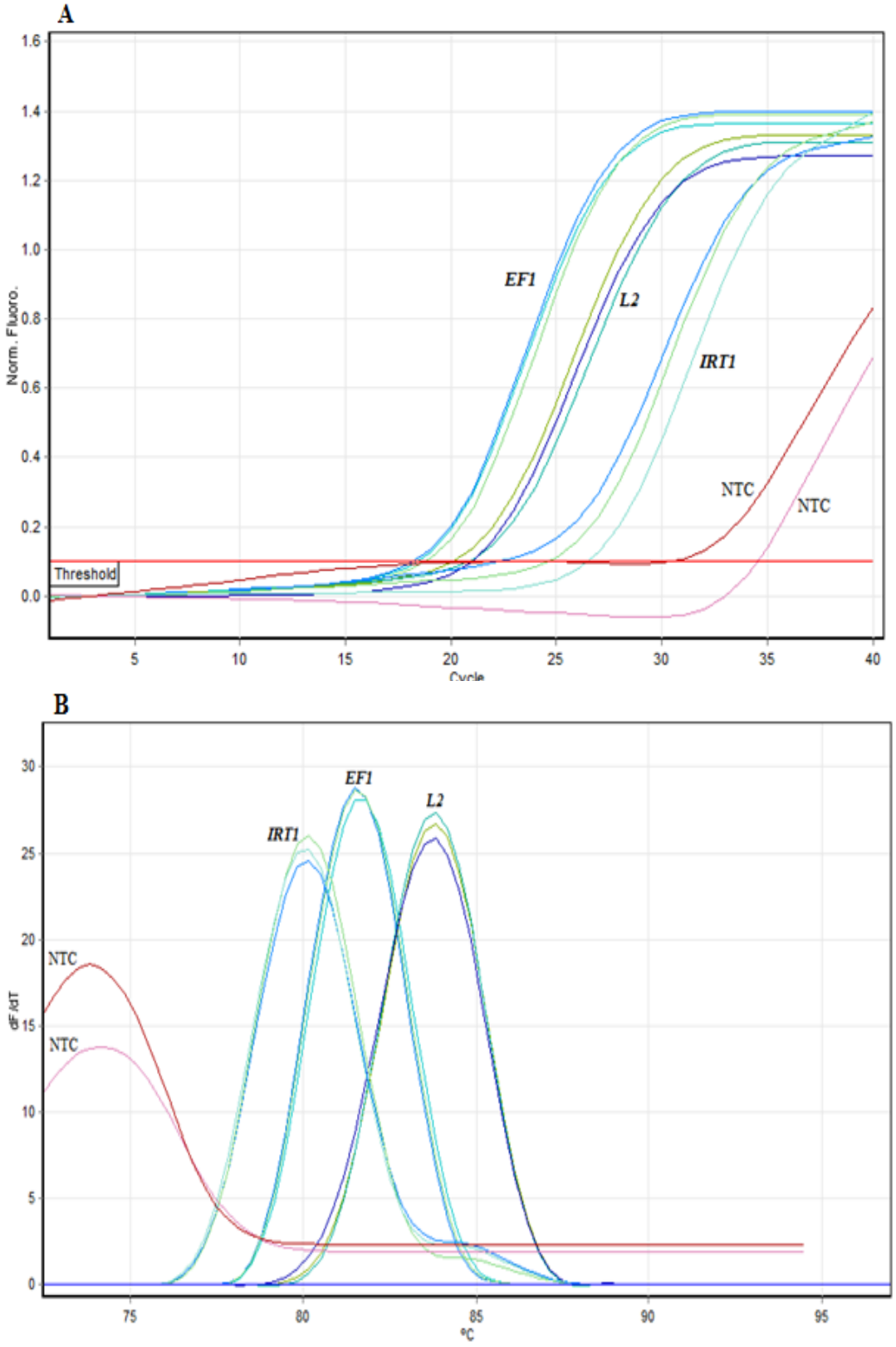


Figure 55. Quality and quantification assessment of cDNA synthesised from potato root and leaf RNA extracts using two reference genes, *L2* and *EF1* genes and a target gene, *IRT1*. A: amplification curve; B: melting curves. Controls without cDNA template (NTC) were also used to determine the specificity of the amplification or template contamination.

6.2.1.3 qPCR primer specificity and annealing temperature

Conditions for RT-qPCRs were optimised with varying primer annealing temperatures. Real-time PCR primers (see Table 15) ordered were as designed by Legay et al. (2012) and Nicot et al. (2005) using a standard set of criteria (see section 2.7.3). Optimisation of primers was done by determining the best annealing temperature (Ta) of the primers for target and reference genes. Ta of a primer pair should generally be 5^{o}C lower than its melting temperature (Tm) hence, Ta range of 58-62^{o}C was evaluated. The 58, 60 and 62^{o}C annealing temperatures used in the qPCR runs produced single discrete peaks of similar products based on the melt and amplification curve data generated. The qPCR oligonucleotide primers were optimised to an equal annealing temperature of 60^{o}C which was used for all further qPCR experiments. The results showed that the most efficient expression occurred at 60°C. Using melting curve data (Figure 56) only a single peak per qPCR primer pair, indicating a single pure PCR amplicon of the desired product melting temperature was displayed. Product-specific melting temperatures were: 81°C (*EF1*), 84°C (*L2*), 80°C (*IRT1*) and 76°C (*FER*). No primer–dimer formations nor non-specific PCR products were detected. A single amplified PCR product (Figure 57) was observed on agarose gel as a single band confirming the specificity of the amplification.

Table 15. Sequences of the oligonucleotide primers used for qPCR**.**

Genes	*Abbr.*	*Accession number*	*Primer Sequence (5'-3')*		*Amplicon size (bp)*	*Tm (°C)*
Ferritin 3	Fer 3	BQ112376.2	Forward:	GGGGAAAATCCGTTAATCCA	64	76
			Reverse:	CACAAATTATCAACCCGTTTCC		
Iron regulated transporter 1	IRT1	BQ514716.1	Forward:	CTTCTTGGTGCTGGTGGAAT	99	80
			Reverse:	GGCGTTGGGTGTGTTTACAT		
Elongation factor 1 alpha	EF1	AB061263	Forward:	ATTGGAAACGGATATGCTCCA	101	81
			Reverse:	TCCTTACCTGAACGCCTGTCA		
Cytoplasmic ribosomal protein L2	L2	39816659	Forward:	GGCGAAATGGGTCGTGTTAT	121	84
			Reverse:	CATTTCTCTCGCCGAAATCG		

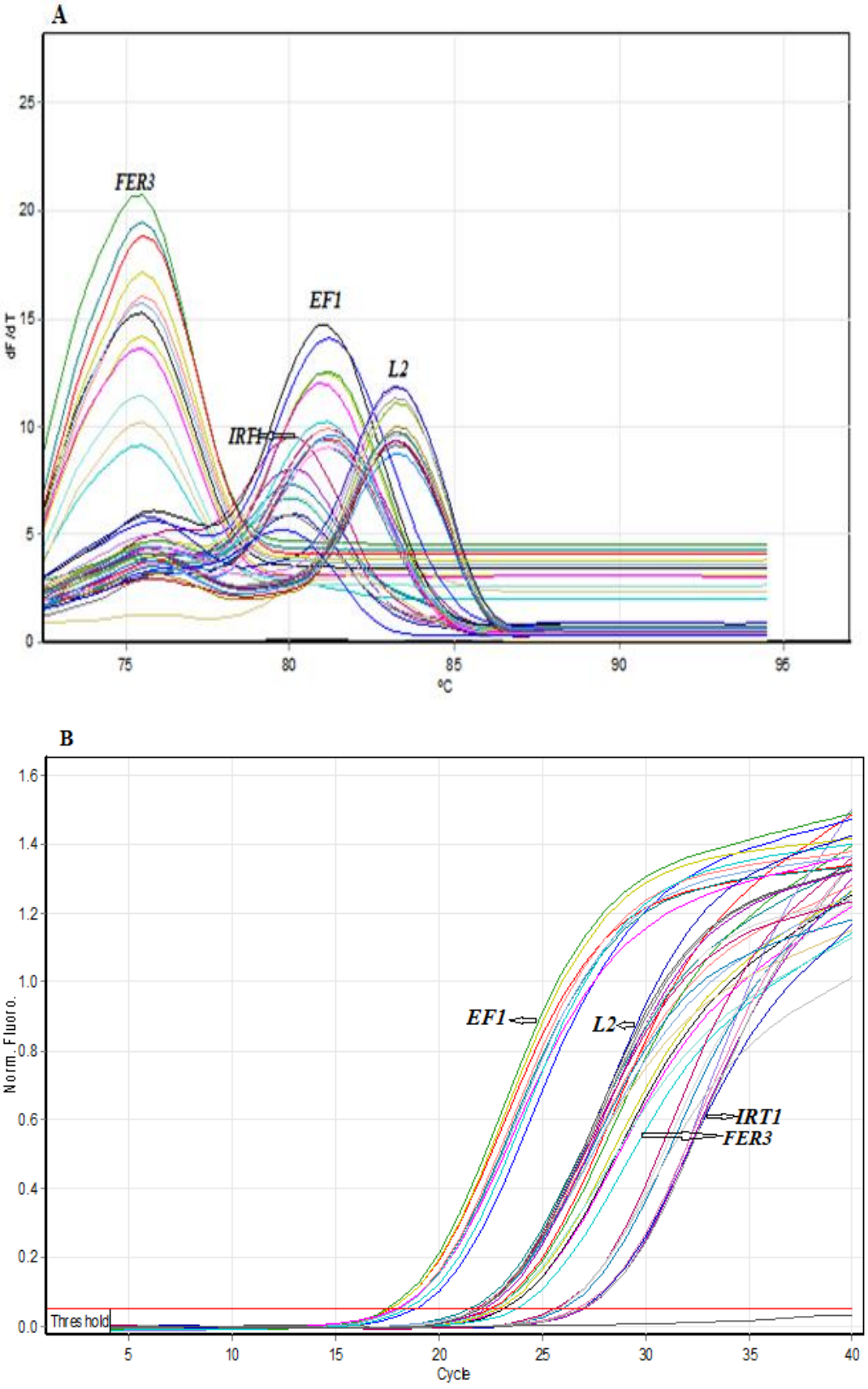


Figure 56. RT-qPCR performed at annealing temperature of 60°C. A. The melt curve and B. amplification profile of products amplified by primers specific for target (*IRT1 and FER3)* and reference genes (*EF1* and *L2).*

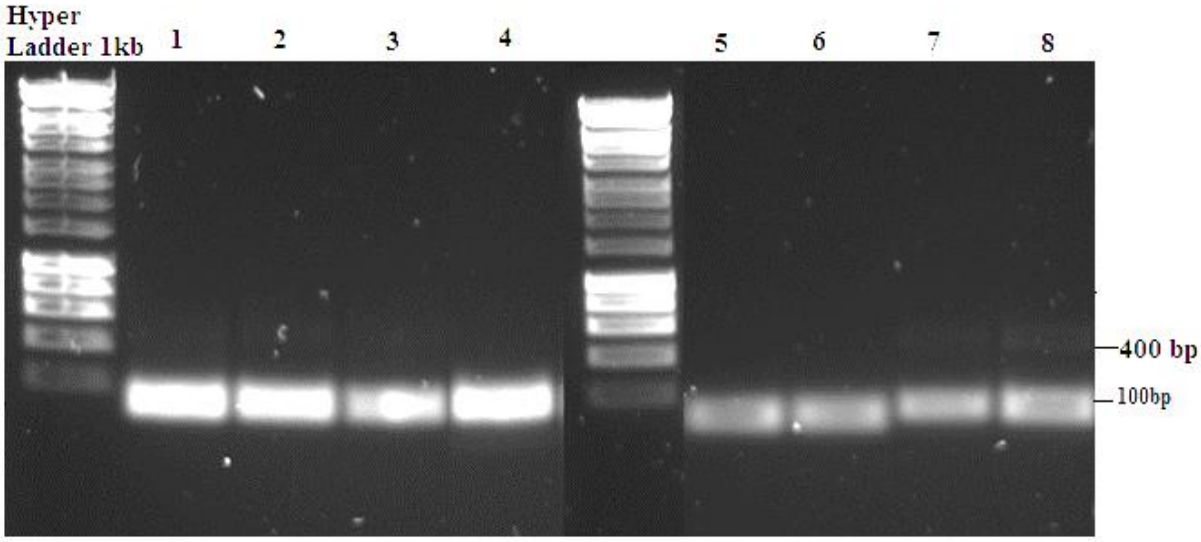


Figure 57. PCR amplicon size analysis on agarose gels. Gel electrophoresis analysis of PCR products amplified by the primers for the reference and target genes confirms the presence of a single specific product. The discrete bands generated are of expected sizes: lanes 1 and 2: EF1 (101bp); 3 and 4: L2 (121bp), 5 and 6: FER3 (64bp); 7 and 8: IRT2 (99bp).

6.2.1.4 qPCR efficiency

The precision of a qPCR assay is dependent upon the efficiency of the reaction. Accurate quantification of gene expression is reliant on high PCR efficiency. All cDNAs were pooled and a standardised 4-fold dilution series was used to assess the PCR efficiency of each gene. The high linearity ($R^2 > 0.99$) of the standard curves (Figure 58) shows that there was a high correlation between cDNA concentration and amplification cycle (Cq). PCR efficiency is close to 100% when the slope of the amplification curve is close to −3.32 which indicates a doubling of PCR product. The results attained showed that the qPCR assays were efficient with slope values of -3.08 (FER3), -2.99 (IRT1), L2 (-3.13) and -3.32 (EF1). An exponential function (E value) of 2 is an indication of 100% amplification efficiency. The E values calculated from the slope were as follows: 2.11 (FER3), 2.15 (IRT1), 2.09 (L2) and 1.99 (EF1). The efficiency of the RT-qPCR reactions was within the acceptable range for a successful assay. Amplifications showed PCR efficiencies in a range of 99.9 to 115%: FER (111%), IRT1 (115%), L2 (1.08) and EF1 (99.9%).

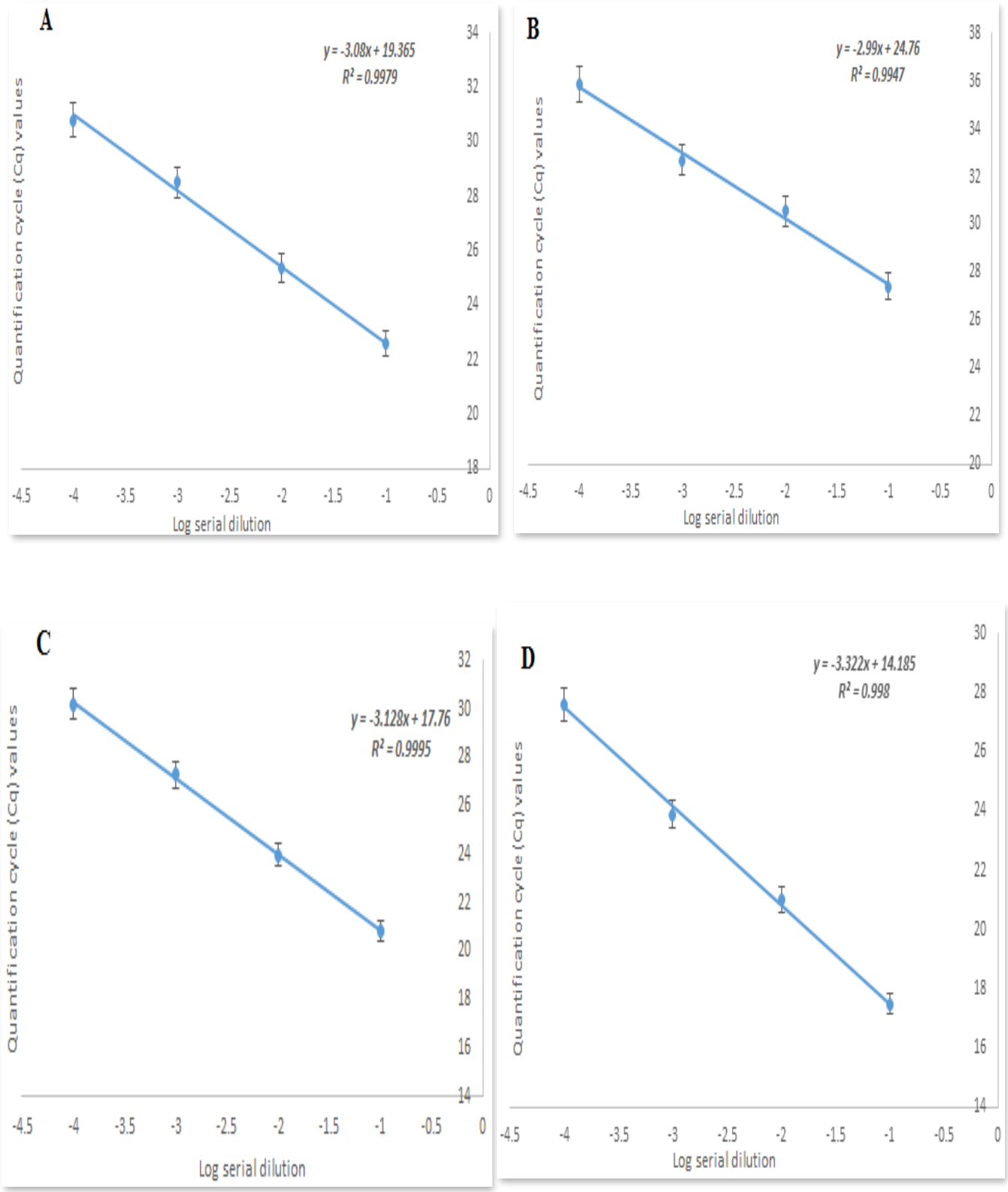


Figure 58. Standard curve of target genes (A: *FER3*; B: *IRT1*) and reference genes (C: *L2*; D: *EF1*) generated from amplification curve data. A 4-point dilution series was created for all genes studied and amplification was performed using KAPA SYBR Master Mix on a Rotor-Gene Q qPCR instrument. Linear regression analysis of standard curves shows high linearity, $R^2 > 0.990$. Cq values are presented as means ± standard error of three independent assays.

6.2.1.5 Validation and normalisation of reference genes

Normalisation of target genes with reference standard is strictly recommended for RT-qPCR analysis (Fleige et al., 2006). Due to sample-to-sample variations and the limitation to control all experimental conditions, validated reference genes are used to evaluate expression of the genes of interest (Fleige and Pfaffl, 2006; Nolan et al., 2006; Udvardi et al., 2008). To normalise the expression of target genes (*FER3* and *IRT1)*, the reference genes, *L2* and *EF1* were used. The reference genes were initially compared for their expression stability in various cDNA samples of leaf and root organs from potato plant lines with differential tolerance to Fe-deficiency. The melting curve data of the two reference genes showed a single peak per gene (Figure 59): *EF1* had a single peak at 81°C and *L2* at 84°C, indicating that only a single PCR product formed. A reference gene should ideally display the same expression level across all samples after normalisation. In the present study, reference gene expression levels varied only slightly (similar mean Cq for each sample). The results (see Figure 59) based on the Cq values of the two reference genes showed that the range of Cq values among the reference genes was narrow with *EF1* having lower mean Cq values (17.1 ± 0.93) and *L2,* maximum Cq values (20.4 ± 1.06). Variation between identical replicates was not greater than ± 1 Cq value. Both reference genes were included in the gene expression studies because the variation in expression was minimal across the different organs (Figure 60). No significant differences in the expression levels between leaf and root organs were detected per endogenous control. For each reference gene, a correction factor (CF) for every cDNA was calculated: the values of four replicates were averaged to form the mean CF for each biological replicate of each reference gene. The final CF value for every biological replicate was evaluated by averaging the CF values of the two reference genes (Dhandapani, 2014; Song et al., 2012).

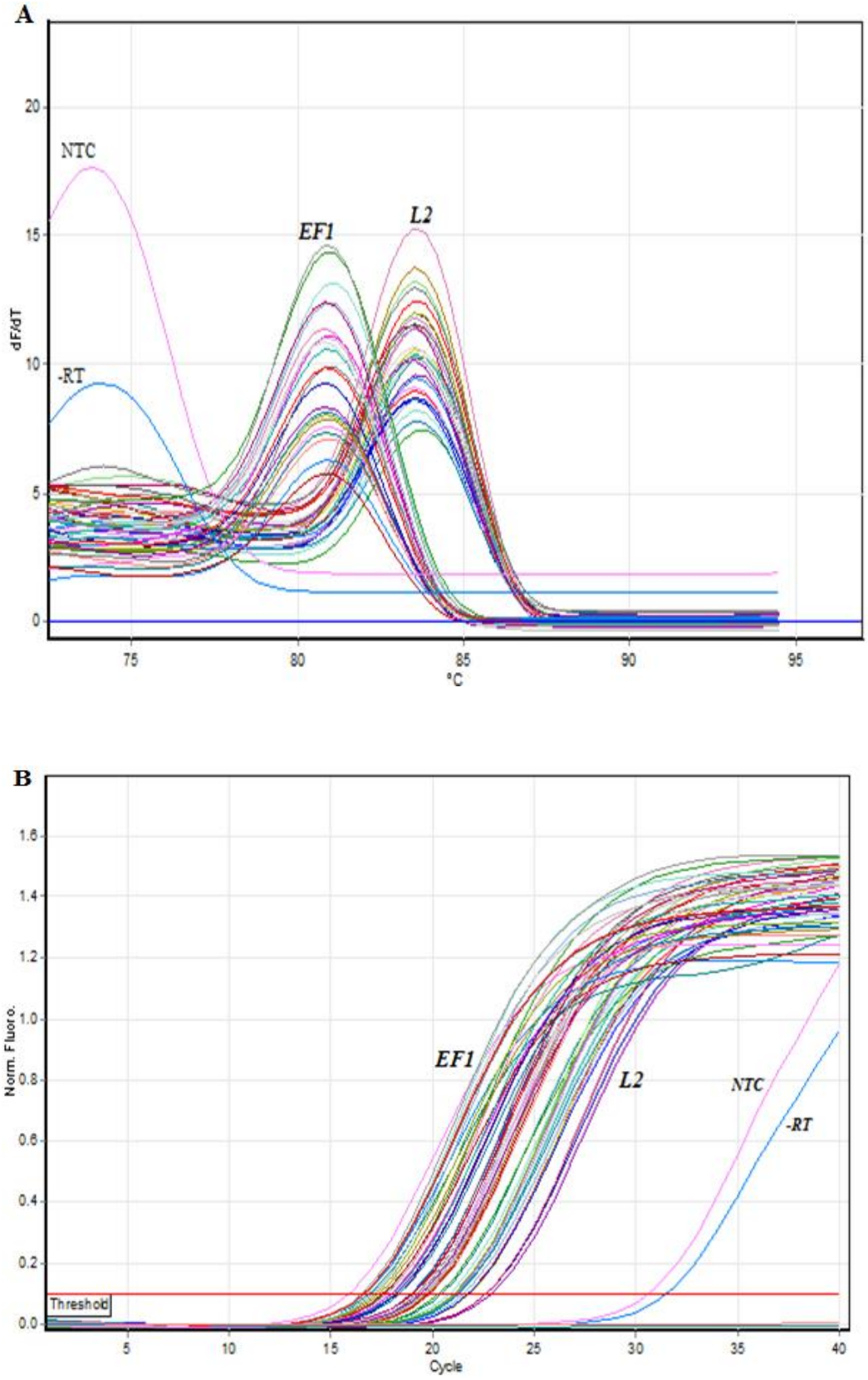
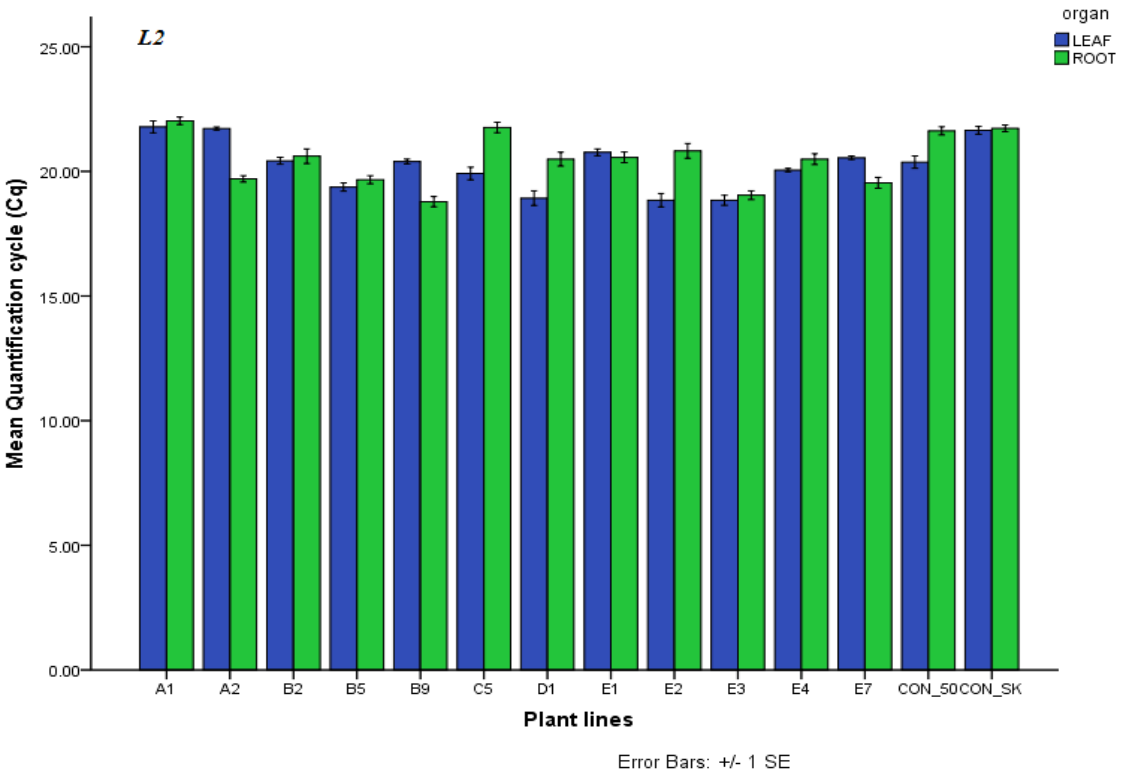


Figure 59. RT-qPCR reaction using primers of reference genes, *L2* and *EF1*. A. melting curve and B. amplification curve of cDNAs of leaf and root samples of potato plant lines (EF, IFN) and control (parental biotype). The negative controls (NTC and -RT) were about 10-15 cycles more than the samples.



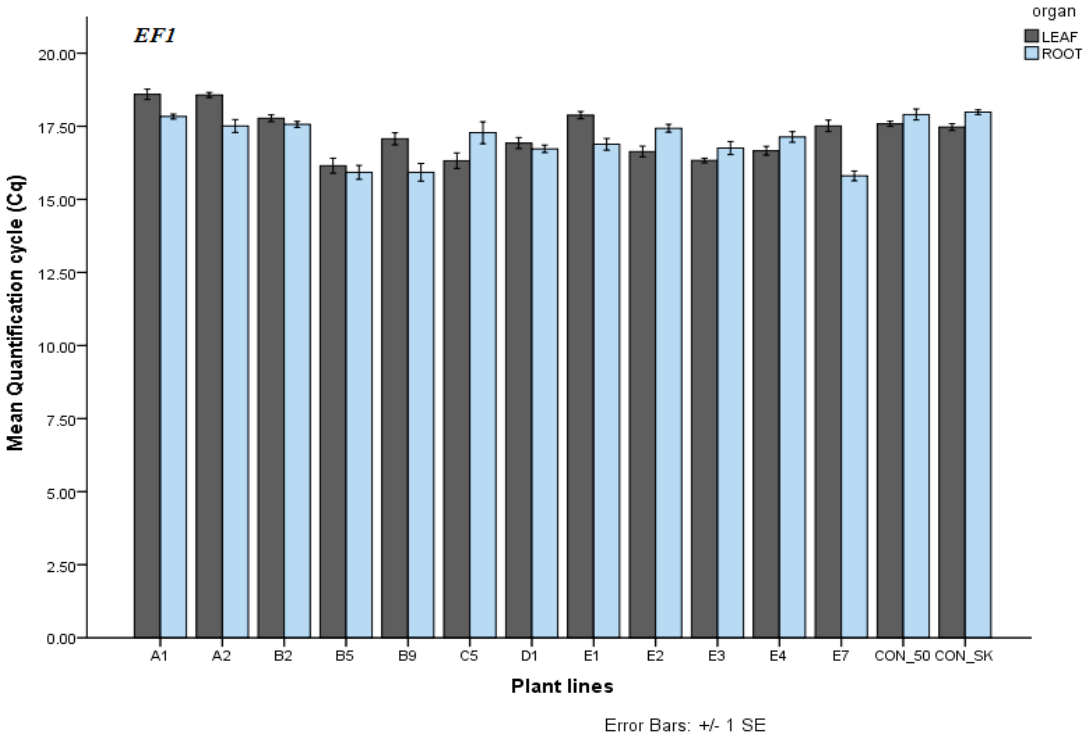


Figure 60. Expression levels (Cq values) of *L2* and *EF1* reference genes in root and leaf of selected Fe-efficient (A1, B2, B9, D1, E1-3, E7), inefficient (A2, B5, C5, E4) potato plant lines and control plants (con-50 and con-SK). Cq values are means three identical replicates ± SE.

6.2.2 Relative quantification of iron-related genes

To gain insight into transcriptional responses of *Solanum tuberosum* (cv "Iwa") plant lines with differential IDC tolerance, expression of iron homeostasis-related genes, *irt1* and *fer3*, was investigated. Using RT-qPCR technique, the relative expression levels of *irt1* and *fer3* were quantified in potato plants lines and control plants (parental stock plant: con-SK and plant regenerated from callus supplied with sufficient, 50µM Fe: con-50). All plants were exposed to conditions of low iron availability for three months prior to RT-qPCR analysis. The cDNAs from leaf and root organs were used for gene expression studies, with the reference genes, *EF1* and *L2*. The cDNAs synthesised from each set of control plants were pooled together to represent as much as possible, the control plant samples to be assessed. Relative quantification of *irt1* and *fer3* expression was calculated taking into account the two reference genes. As explained in section 6.1.4, the expression of the two reference genes was adjusted by calculating a correction factor (CF) for individual cDNAs for each reference gene. The final CF value was calculated by averaging the CF values of the two reference genes and its correlation to the target genes *irt1* and *fer3* was evaluated.

6.2.2.1 Expression of *fer3* involved with Fe-efficiency

Plant ferritin plays an essential role in the maintenance of iron buffering, oxidative stress prevention and adaptation to adverse environmental situations (Parveen et al., 2016; Briat et al., 2010; Ravet et al., 2009). To investigate the molecular basis for differential Fe-efficiency between potato plant lines, the expression of ferritin gene, associated with iron storage in plants was studied. The normalised relative expression levels of *fer3* in various plant lines are presented in Figure 61. Overall, the expression profile of *fer3* varied among plant leaf and root organs (Figure 61). *FER3* expression levels were highest in leaves of the potential Fe-efficient line, E7, but its expression in E7 roots was reduced in relation to the control plants. Conversely, the relative expression of *fer3* was significantly increased in the root of the Fe-inefficient line, C5 (16. 86-fold change) but considerably decreased in C5 leaf (6.47 fold change). In the IFN line, B5 however, *fer3* expression was consistently lowest in both leaf (3.20 fold change) and root (2.19 fold change). With A1 and E1 putative Fe-efficient plant lines, *fer3* expression in both leaves and roots were significantly increased compared to the parental biotype (con-SK). In all, the results revealed that under Fe-deficiency conditions, *fer3* was expressed to a significantly greater magnitude in leaves than in roots by 38% ($t = 4.798$, $df = 222$, $p<0.05$).

a) Leaf *fer3* expression

About 90% of the Fe-efficient plant lines had increased leaf *fer3* expression levels relative to the con-50 control plant ($p<0.05$) as shown in Figure 61. Approximately 62% of the EF lines (E7, B2, B9, E1, A1) had significantly higher *fer3* expression levels compared to both control plants (con-SK and con-50). The least *fer3* expression was detected in the leaves of the IFN lines, C5 and B5 (3.20 and 6.46-fold change) whereas peak levels (22.60 – 26.41-fold change) of *fer3* transcripts were detected in the leaves of B2, B9, E1 and E7 potential Fe-efficient lines.

The results presented herein indicate different responses among leaves of potato plant lines at the *fer3* gene expression level, which may determine their tolerance to IDC. The expression profile of *fer3* in the leaves of predominantly IDC tolerant plant lines was found to be significantly higher compared to IDC sensitive plants. A plausible explanation for this result could be that the induction of *fer3* expression due to low Fe availability possibly serves as a response mechanism to improve Fe acquisition in IDC tolerant plants. The findings suggest that Fe-efficiency may be linked to enhanced *fer3* expression which serves to facilitate the Fe acquisition and/ or storage capacity of Fe-efficient plants probably as an adaptive mechanism to enable survival under Fe-deficiency stress conditions. According to Zok et al. (2010) increased production of ferritin improved abiotic stress tolerance in transgenic grapevine plants. Transgenic plants characterised as ferritin (*fer1*) overexpressors in chickpea conferred enhanced growth and altered expression of iron-related genes (Parveen et al., 2016). High expression of leaf *fer3* perhaps confers tolerance to IDC in potato.

Ferritin gene expression at the leaf level is proposed to be transcriptionally regulated by iron. Parveen et al. (2016) has reported on differential accumulation of ferritin **(***fer1*) transcript in chickpea seedlings in response to moderate and excess amounts of Fe. Legay et al. (2012) showed that ferritin expression in leaves of potato plants grown on high Fe medium displayed increased *fer3* expression while expression decreased in plantlets grown in a low iron medium. Likewise, excess Fe caused a significant increase in *fer1* transcript levels in Arabidopsis leaves (Reyt et al., 2015). Earlier studies reported that Fe overload activates the expression of ferritin, which sequesters Fe in a non-toxic form (Kobayashi et al., 2012; Briat et al., 2010). In iron-deficient plants, it has been suggested that unavailability of catalytically active Fe might be due to increased expression of ferritin (Graziano and Lamattina, 2007; Tewari et al., 2013).

The results herein provide insight into the role of the *fer3* gene in maintaining iron homeostasis within plants while possibly transporting iron to the chloroplasts where the demand for Fe (for photosynthesis and/or chlorophyll synthesis) is high. Elevated *fer3* expression in the putative Fe-efficient lines may be connected to the high leaf chlorophyll content (see Figure 44) detected in these plant lines. Ferritin proteins store Fe chiefly in the chloroplasts although some can be found in the mitochondria and other plastids (Briat et al., 2010). Perls staining has been useful in the identification of Fe-ferritin in the chloroplast of mesophyll cells (Roschzttardtz et al., 2013).

b) Root *fer3* expression

The results given in Figure 61 portrays that the *fer3* gene was highly expressed in the roots of 37.5% of the potential Fe-efficient plant lines (i.e. A1, E2 and E1) relative to the parental biotype (con-SK). Root *fer3* expression was highest in the A1 Fe-efficient line with the relative level of expression increased by 23% and 53% compared to con-50 and con-SK control plants respectively. In 50 % of the Fe-efficient lines (D1, B2, E7, E3), relative root *fer3* expression (6-8 fold change) was the same as that of con-SK but significantly reduced (2 -3 times less) with regards to the con-50. Interestingly, the C5 Fe-inefficient line, which developed severe chlorosis (see Table 9), showed elevated root *fer3* expression compared to the control plants and other potential Fe-efficient lines except A1.

Unlike in leaf, the functional role of ferritin or its involvement in oxidative stress responses in roots has been studied less thoroughly and some findings are debatable (Reyt et al., 2015; Vignani et al., 2013). The results of the current study revealed that the level of *fer3* transcripts was strongly increased in the roots of both sets of plant lines and control plants exposed to Fe deficiency. Inversely, a marked reduction in *fer3* expression was observed in other Fe-efficient and inefficient plant lines. It appears that at the root level, differential tolerance to IDC in potato plants may not be linked to *fer3* expression levels. However, Santo et al. (2015) have reported that Fe-inefficient plants (at an early growth stage) had higher induction levels of the *ferritin* gene in the roots than Fe-efficient plants. The authors argued that the high level of ferritin expression by the roots of soybean IFN plants could be responsible for the accrual of Fe with ferritin in roots and, as a result, made Fe part itioning to the shoots more difficult.

A likely role of Fe in the localisation and regulation of root ferritin expression has been documented by other researchers. Vignani et al. (2013) reported on the localisation of the ferritin in mitochondria of cucumber roots. The authors revealed that ferritin abundance was strictly dependent on the amount of Fe in mitochondria which, in turn, was reliant on Fe availability in the growth medium. Ferritin was found to be abundant in root mitochondria of Fe-excess plants with lower detection levels in roots of plants grown in control Fe medium but not in Fe-deficient ones (Vignani et al., 2013). Reyt et al. (2015) reported that the ferritin gene, *fer1*, was most highly expressed in Arabidopsis roots in response to excess Fe. Low-iron availability led to the repression of *fer3* expression in the roots of potato plantlets whereas in high Fe medium, *fer3* expression was significantly high (Legay et al., 2012). According to Vignani et al. (2013), low Fe levels in the mitochondria could activate the repression of ferritin synthesis to prevent further depletion of Fe by ferritin, since Fe is needed for the assembly of the respiratory units.

Studies on the functions and regulation of plant ferritin have revealed associations between ferritin and protection against oxidative stress (Briat et al., 2010; Parveen et al., 2016). Excess Fe conditions or mutations resulting in high Fe levels in plants caused an increase in ROS production and elevation of oxidative stress responses (Parveen et al., 2016; Reyt et al., 2015; Briat et al., 2010; Ravet et al., 2009). In such situations, ferritin accumulated in chloroplasts (Briat et al., 2010) and functioned in buffering Fe to preventing oxidative stress (Ravet et al., 2009). Perhaps the improved antioxidant capacity (elevated POD activity) exhibited by the selected Fe-efficient lines (see section 5.5.4 and Figure 48) could be linked to the high *fer3* expression detected mainly at the leaf level. Parveen et al. (2016) suggests that ferritin (*fer1*) is responsible for Fe-mediated oxidative stress tolerance in chickpea. The reduction in H_2O_2 stains detected in CaFer1 transgenic leaves compared to wildtype was attributed to ferritin-mediated reduction of ROS.

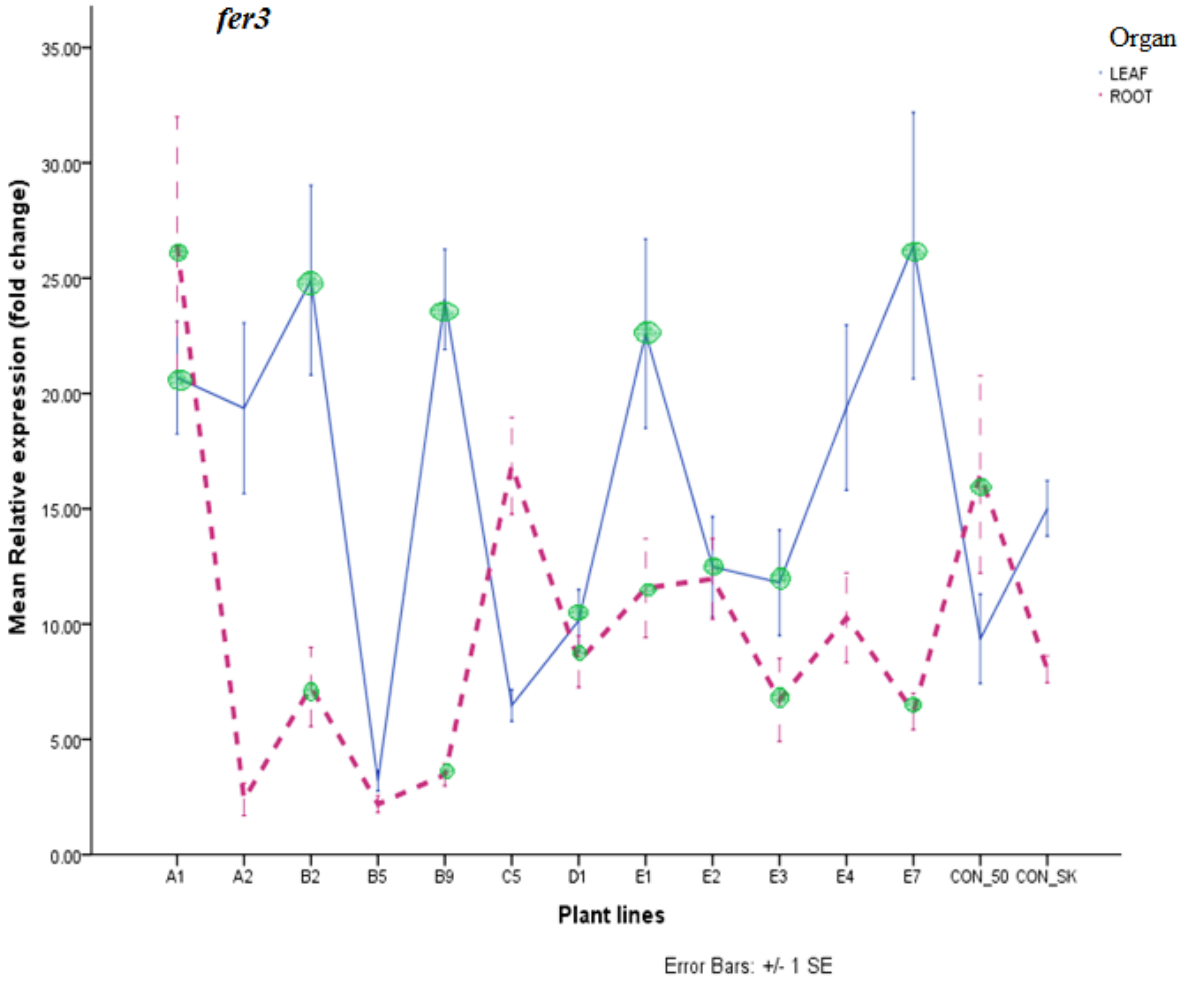


Figure 61. Normalised relative expressions of *fer3* in root and leaf of *Solanum tuberosum* plants with differential tolerance to Fe-deficiency induced chlorosis. Plants were exposed to Fe-deficiency conditions for three months. Values are means of relative transcript levels (in fold change) of four replicates for each of two biological replicates (n=8). *EF1* and *L2* reference genes were used to normalise *fer3* expression levels. Error bars represent the ± standard error (SE) of the mean calculated for the combined sample and biological replicates. Highlighted data points (green circles) represent plant lines selected to be potentially Fe-efficient based on IDC scores ≤ 2.42.

6.2.2.2 Fe-deficiency induced changes in *irt1* expression

The Fe-regulated transporter, IRT1, has been proposed to be upregulated in potato and other Strategy 1 plants in response to Fe deficiency (Legay et al., 2012; Henriques et al., 2002; Vert et al., 2002). In the current study, the molecular effect of Fe deficiency on *irt1* was examined in roots and leaves of chlorosis tolerant and sensitive potato plants. The results (Figure 62) show that Fe deficiency induced changes in *irt1* transcript levels in largely roots of plants with differential tolerance to Fe-deficiency and confirm that *irt1* has a role in Fe uptake in potato. The pattern of *irt1* expression in leaf was different to that of root with a much lower

range of expression in the leaves. *IRT1* expression was elevated in roots than in leaves by 20% with the difference (19.87 fold change) in the mean *irt1* relative expression being statistically significant ($t=-3.24$, $df=222$, $p<0.05$). In the A1 Fe-efficient line however, *irt1* expression in the leaf (224 fold change) was comparable to that in the root (211 fold change) and both values were significantly increased compared to the control plants.

a) Leaf *irt1* expression

The results presented in Figure 62 reveal that *irt1* was most highly expressed in the putative Fe-efficient line, A1. The increase in A1 leaf *irt1* expression was twice as much as that of the parental biotype, con-SK and 14-fold greater than con-50. Among the IDC sensitive lines, *irt1* expression was highest in the leaves of A2 with expression levels 3.3-fold more than that of con-50 plants but lower than con-SK by 2.1-folds. Expression of *irt1* was high in the leaves of 62% of the putative EF lines and 50% of the IFN plant lines with respect to the con-50 control plant but the change in expression was not statistically significant ($p>0.05$). *IRT1* expression in the leaves of about 83% of all plant lines was significantly low compared to the parental biotype, con-SK.

Literature on *irt1*expression in leaves is scanty but earlier studies by Vert et al. (2002) have shown that *irt1* expression is noticeable in leaves and not restricted to the roots. The *irt1* gene was detected in rosette leaves and also found to be expressed in the basal part of flowers, suggesting its role in Fe acquisition in aerial tissues. In the current study, *irt1* expression in leaves was generally low but significantly high levels were identified in the putative A1 Fe-efficient line, the A2 Fe-inefficient line and the parental control plant. Similarly, Santos et al. (2015) indicated that IRT1-like gene expression was high in leaves of both EF and IFN plant lines in Fe-liming conditions. The authors suggested that the removal of unifoliate leaves in EF plants caused the accumulation of more Fe in the aboveground organs and accounted for the increased expression of *IRT1*-like gene in the shoots. In examining plants' response to Fe supply, it was observed that *irt1* mRNA was reduced in leaves of plants grown in iron-limited conditions (Legay et al., 2012; Barberon et al., 2011).

Vert et al. (2002) observed severe leaf chlorosis typical of iron deficient plants in the Arabidopsis knockout mutant in *IRT1, irt1-1*. Iron supplementation in the *irt1-1* plants resulted in green and healthy plants indicating that iron is required for chlorophyll biosynthesis. In the present work, not enough evidence has been found to imply that high chlorophyll content in leaves of potential Fe-efficient lines (as discussed in Section 5.5.1)

may be directly related to the high expression of *irt1* in leaves. This pinpoints a possible lack of correlation between visual chlorosis symptoms and chlorophyll content of the potato plant lines and their molecular characteristics relating to leaf iron transport.

b) Root *irt1*expression

The results in Figure 62 depict elevated *irt1* expression in the roots of 50% of the putative EF lines (A1, B2, B9, E3) and control plants. Relative to the parental biotype (con-SK), *irt1* expression was increased in 25% (A1, B9) of the Fe-efficient plant lines. The expression of *irt1* was highest in the putative A1 Fe-efficient plant. The level of *irt1* transcripts in A1 roots was significantly increased by 25% and 44% relative to con-50 and con-SK respectively (Figure 62). In B9 root however, *irt1* expression peaked (by 9.16 fold change) against the con-SK but decreased (by 35.25 fold change) in relation to the con-50 plant. In the other 75% of the plant lines, root *irt1* expression was significantly low. Root *irt1* expression level was low in all IFN plant lines compared to the control plants ($p<0.05$). Surprisingly, the roots of some EF plant lines (E1-2, E7) had the least *irt1* expression levels.

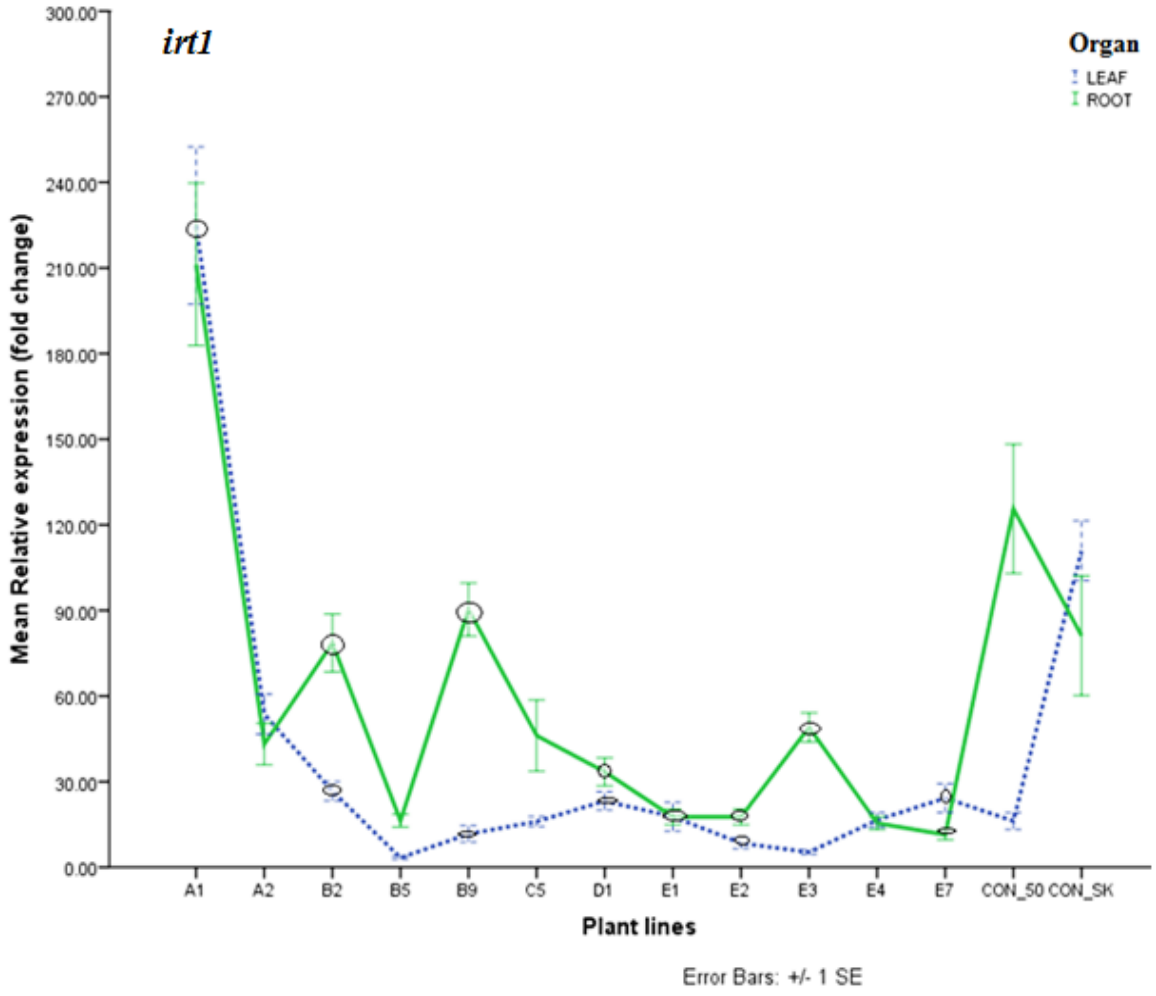


Figure 62. Normalised relative expressions of *irt1* in root and leaf of *Solanum tuberosum* plants with differential IDC tolerance. Plants were exposed to Fe-deficiency conditions for three months. Values are means of relative transcript levels (in fold change) of four replicates for each of two biological replicates (n=8). *EF1* and *L2* reference genes were used to normalise *irt1* expression levels. Error bars represent the ± standard error (SE) of the mean calculated for the combined sample and biological replicates. Highlighted (green circles) data points represent plant lines selected to be potentially Fe-efficient based on IDC scores ≤ 2.41.

IRT1 is expressed predominantly in the epidermis of roots and is activated by iron deficiency (Vert et al., 2002; Hindt and Guerinot, 2012). This may account for the significantly higher *irt1* expression levels detected in roots than in leaves in the present study. This observation may be an indication that *irt1* plays a vital role in the response of potato plants exposed to Fe-deficiency conditions by enhancing Fe-transport capacity, mainly in the roots in agreement with previous reports (Vert et al., 2002; Legay et al., 2012; Kabir et al., 2015). Legay et al.

(2012) showed that *irt1* expression in the root was regulated by the amount of Fe supplied to growth medium. The authors observed a strong increase in *irt1* expression in roots of plants grown in low Fe medium but lowered expression under high Fe supplies. This suggested that IRT1 was involved in iron homeostasis in potato plants.

The results presented herein reveal that *irt1* expression in the roots of 50% of the potential Fe-efficient plant lines was enhanced as a consequence of Fe deficiency (see Figure 62). Additionally, *irt1* was expressed at increased levels in the roots of both IDC tolerant and sensitive potato plant lines with a more pronounced effect detected in the IDC tolerant line, A1. These findings are consistent with earlier reports on *irt1* expression in plants with differential IDC tolerance. The expression of *irt1* was induced in the roots of chlorosis tolerant and sensitive okra genotypes due to Fe deficiency but the expression was higher in the tolerant genotype (Kabir et al. 2015). The findings of the present study could not establish specifically that Fe-efficiency might be attributable to higher root *irt1* expression because significantly increased (50% EF lines) and decreased (50% EF lines) *irt1* expressions were displayed by the putative IDC tolerant lines. Furthermore, some chlorosis sensitive lines (A2, C5) showed greater *irt1* expression levels than other tolerant lines (E1-2, E7) similar to findings of Martínez-Cuenca et al. (2013) which indicated that chlorosis sensitive citrus rootstocks showed the greatest (60.1%) induction of *irt1* expression than the tolerant ones (26.7%). Furthermore, Santos et al. (2015) noted that the expression of IRT1-like gene was higher in the roots of IFN plants than in the EF ones.

IRT1 expression in the potato plants used in the current work may have been influenced by the presence of other minerals such as Zn, Mn, Co and Cu normally present in the plant growth medium since these metals may have been preferentially transported by *irt1* under Fe-deficient conditions. Vert et al. (2002) found that Zn, Mn and Co increased intensely in the roots of plants grown under iron deficiency. The authors suggested that the IRT1-mediated accumulation of Zn, Mn and Co may be physiologically significant because such metals are known to substitute iron in some cellular processes under low-iron conditions. Although IRT1 has a high affinity for Fe^{2+} (Vert et al., 2002; Henriques et al. 2002), it can trigger accumulation of other minerals since it also facilitates the transport of Zn, Mn, Cu and other heavy metals that accrue in plants during Fe starvation (Korshunova et al., 1999; Cohen et al., 1998; Hindt and Guerinot, 2012). A report by Barberon et al. (2011) indicates that *IRT1* overexpression led to strong accumulation of metals in plants.

There have been conflicting reports and divergent conclusions concerning the molecular responses of EF and IFN plants under Fe limiting conditions: some studies (Kabir

et al., 2015) have shown that in EF plants, the expression of Fe-deficiency related genes was increased while others (Martínez-Cuenca et al., 2013; Santos et al., 2015) have detected enhanced expression of these genes in IFN plants. Santos et al. (2015) argued that IFN plants (at an early growth stage) having been more severely affected by Fe shortage than the EF plants, had the necessity to induce Fe-uptake related genes.

6.3 Summary

The normalised relative expression profile of *fer3* and *irt1* varied among plant leaf and root organs. In the current study, ferritin expression levels were more pronounced (by 38%) in leaves than in roots in accordance with reports in literature which indicate that ferritin is localised primarily in leaf in chloroplasts where photosynthesis is active (Darbani et al., 2013; Briat et al., 2010, 2006). *IRT1* expression levels were 20% greater in roots than in leaves. This is consistent with previous findings that *IRT1* expression is induced by iron deficiency largely in the roots (Vert et al., 2002; Hindt and Guerinot, 2012). *IRT1* expression in the roots of 50% of the potential Fe-efficient plant lines (A1, B2, B9, E3) was enhanced in response to Fe deficiency. Relative leaf *irt1* expression was largely low in all plant lines compared to the control plants except for the IDC tolerant line, A1, which showed a marked rise in *irt1* mRNA levels. The considerably high gene expression levels (mainly *irt1)* measured in control plant roots and leaves may have been due to rapid response to Fe deficiency stress upon initial exposure following continued growth in sufficient Fe medium compared to the plant lines which had been derived from callus cells exposed to long-term Fe deficiency conditions.

The A1 chlorosis tolerant plant exhibited characteristic Fe-efficiency responses in leaf and root organs at the molecular level (increased *fer3* and *irt1* transcripts). High *IRT1* expression in roots may have led to build-up of the iron-binding protein ferritin in leaves similar to findings by Barberon et al. (2011). Other putative EF plant lines (B2, B9, E2, E1 and E7) displayed significantly high *fer3* and /or *irt1* expression levels either in leaf and/or root organs. Contrarily, *fer3* and /or irt1 were expressed highly in some IFN lines compared other EF lines in agreement with results of other studies (Martínez-Cuenca et al., 2013; Santos et al., 2015).

The research findings presented herein provide insight into molecular responses influencing homeostasis of Fe nutrition in potato plants and a probable coordinated role of *fer3* and *irt1* genes in Fe acquisition under Fe-deficiency conditions. Taken together, the results show that *fer3* (for Fe storage) and *irt1* (for Fe transport) play essential roles in iron homeostasis principally in the leaves and roots respectively. Root IRT1 and leaf FER3 appear to be linked to Fe-efficiency trait in potato. These molecular studies can contribute to the advancement of a broad understanding of Fe homeostasis in plants, which can support efforts to develop crop varieties with enhanced tolerance to iron-limiting growth conditions and improved sustainability in agriculture. Such plants may be possible candidates for improving iron levels in plants with implications of reductions in the incidence of iron deficiency in humans.

CHAPTER SEVEN

Conclusions and recommendations

7.0 Introduction

There is no prior study of using *in vitro* culture technology for the selection of Fe-efficient plant lines in potato (*Solanum tuberosum* cv 'Iwa'). The main aim of this study was to investigate the effectiveness of *in vitro* plant tissue culture as a tool for obtaining Fe-efficient potato plant lines and to characterise the selected lines. The selection for Fe-efficiency was accomplished based on selection against Fe deficiency in callus cultures initiated from leaf explants of 'Iwa' potato. Since plants may not regenerate from such cultures and/or the trait of interest may not be expressed at the plant level, this research pursued the regeneration of plants from Fe-efficient potato calli and examined whether the characteristics associated with Fe-efficiency expressed in the selected calli could also persist in potato plants regenerated thereof. This study has confirmed the potential of using callus culture to regenerate plantlets and to establish plant lines useful for examining the persistence of the Fe-efficiency trait in potato.

The knowledge gaps in the responses of potato callus to Fe deficiency and characteristics associated with chlorosis tolerance in potato appear to be impediments to devising approaches to improve plant growth in soils with minimal Fe content. Information on the responses of potato calli and regenerants to Fe-deficiency will aid in broadening our understanding of the mechanisms that control Fe acquisition and utilisation in potato. This study plays an important role in generating novel materials that provided a means of delineating the responses associated with Fe uptake, transport and localisation in potato under Fe-deficiency conditions. The knowledge gained can be translated to other plants. A major objective of this study was to assess the morphological, biochemical and molecular responses of calli and to characterise selected Fe-efficient plants. To achieve this, fresh weight, area of growth covered and visual chlorosis symptoms were assessed. The activity of ferric chelate reductase which is involved in the binding and uptake of Fe was measured. Furthermore, antioxidant enzyme activities, phenolic content and photosynthetic pigments that can be influenced in response to iron deficiency were quantified. Transcript levels of key genes (ferritin and iron-regulated transporter) associated with iron transport and homeostasis in plants were evaluated by means of real-time RT-PCR analysis.

The efficient protocol developed in this study for the *in vitro* selection of Fe-efficient potato cell lines may well impact on future research designs in the selection for chlorosis

tolerance in other plants. Selection of iron-efficient potato cell lines using *in-vitro* culture techniques can play a vital role in establishing different lines to aid a better understanding of iron nutrition in potato. In the following sections, the main findings presented in Chapters three to six were summarised and their implications, possible applications of the method and recommendations for future research were discussed.

7.1 *In vitro* selection of Fe-efficient (IDC-tolerant) callus cells

This study demonstrates that the use of *in vitro* plant tissue culture offers a powerful tool and is an efficient method for the selection for Fe-efficiency in both callus cultures and in the regenerated plants. Effective *in vitro* protocols for the selection for Fe-efficiency at the cellular and plant level have been designed. A two-step direct selection scheme employing Fe-deficiency as selective pressure was successful in inducing and generating novel putative Fe-efficient somaclonal variants at the callus level.

The plant regeneration procedure applied was effective in deriving plantlets from IDC tolerant calli. A new set of potato plant lines has been successfully established from regenerants derived from the selected Fe-efficient calli. The confirmation of the Fe-efficiency status of the cell lines generated has been achieved through the subjection of regenerants to the Fe-deficiency selective pressures employed at the callus level. Plant lines established following plant regeneration from the selected callus cultures were distinguished into EF and IFN based on low and high chlorosis scores respectively. A unique set of eight Fe-efficient and twenty-seven inefficient potato lines have been identified and their tolerance to Fe deficiency chlorosis was established under *in vitro* culture conditions. Taken together, somaclonal variation seems to underpin the differential response of calli and/or plant lines subjected to the same Fe deficiency condition.

7.2 Responses of callus cultures to Fe deficiency

An Fe-efficient plant is able to grow in Fe deficient environments through the activation of specific Fe-stress responses that facilitate the uptake of Fe from the soil in a soluble usable form (García-Mina et al., 2013). It is suggested that the key characteristics exhibited by Fe-efficient variant potato cell lines were those that enabled them to effectively utilise Fe from growth medium and enhance tolerance to Fe shortage. The responses of potato callus cultures to Fe supply over a short-term and extended period in culture have been delineated using calli

produced in this study. The morphological and biochemical responses that can serve as indicators of the Fe nutritional status of calli are presented. The morphological responses include visual chlorotic symptoms (yellowing), reduced fresh weight and area of callus growth. Fe shortage was linked to a decrease in chlorophyll and carotenoid content, reduction of antioxidant (POD, CAT and APX) enzyme activities and an increase in lipid peroxidation response in calli. Moreover, exposure of potato calli to Fe deficiency enhanced ferric reduction activity, induced phenolic production and increased H_2O_2 generation. Whereas Fe distribution in cells of Fe-deficient calli was sparse, Fe was widely distributed among cells of calli cultured under sufficient Fe conditions.

The morphological and biochemical responses assessed were mainly pronounced with prolonged exposure to Fe deficiency leading to senescence or death of cells especially in chlorosis susceptible calli whereas chlorosis tolerant cells maintained greenness and viability. It was found that exposure to an extended period of Fe-deficiency stress condition was more effective in clearly discriminating between putative tolerant and sensitive somaclonal variants.

7.3 Characteristics of potato plant lines under Fe-deficiency conditions

This study has provided insight into iron deficiency induced responses that are useful for the characterisation of selected lines in *S. tuberosum* plantlets. The characteristics that enable Fe-efficient plant lines to survive under Fe-limiting conditions were presented. Examination of the responses of potato plant lines to Fe-deficiency was beneficial for gaining understanding of their differential tolerance to IDC. Overall, Fe-efficient plant lines exhibited differential biochemical, morphological and molecular responses as a consequence of Fe-deficiency compared to inefficient lines.

Differential chlorosis susceptibility in potato was linked to specific morphological parameters. Plants affected by IDC (inefficient lines) were sensitive to Fe deficiency and developed interveinal chlorosis while the Fe-efficient lines mostly had green young leaves and were non-chlorotic under Fe-limiting conditions. The Fe-efficient potato plant lines identified had a great potential for tolerance to chlorosis and could thrive under conditions of low-Fe availability unlike inefficient plants and or plant lines which were negatively affected or even die due to sensitivity to Fe shortage. The findings on morphological characteristics of plant lines suggest that EF plant lines are more tolerant to Fe deficiency than control plants and some IFN lines by maintaining an enhanced formation of root hairs and lateral root

formation, shorter root lengths and stem heights. Also, Fe-efficient lines possessed shorter internodes and had increased leaf number per plant. The development of more lateral roots and root hairs in Fe-efficient lines was a characteristic Fe-efficiency response which possibly enabled the effective exploration of the medium for the acquisition of Fe. Shorter shoots and root lengths may be morphological adaptations or cost of fitness to Fe deficiency tolerance. This would possibly ensure that efficient and/or even distribution of the minimal amounts of Fe available over a reasonably reduced distance or surface area.

The examination of the possible biochemical responses by which leaves and roots of potato plant lines cope with Fe deficiency was vital to gaining insight about the tolerance mechanisms. Differences in chlorophyll, carotenoid and phenolic concentrations, POD and FCR enzyme activities were found among EF and IFN lines relative to control plants. The present data suggest that the efficient utilisation of Fe for the synthesis of chlorophyll together with the effective control of oxidative stress and Fe reductase activity may account for the higher tolerance of Fe-efficient plant lines to Fe deficiency as compared to inefficient ones. The sustained growth of chlorosis tolerant lines under Fe deficiency conditions could be partly explained by their abilities to stimulate FCR and POD enzyme activities in conjunction with improved leaf photosynthetic capacity. This study has shown that photosynthetic pigment content, FCR activity and POD antioxidant enzyme activity could be used as biochemical indicators of Fe deficiency tolerance in potato.

The variability of tolerance to Fe deficiency between the two sets of potato plant lines reflects the differences in root responses. Roots of Fe-efficient plants showed increased ferric reductase activity, decreased chlorophyll and carotenoid content and elevated POD activity. The reverse was observed in the roots of inefficient plant lines. Similarly, leaves of the plant lines generated showed different biochemical behaviours under Fe starvation, which may be a reflection of genotypic differences. The results suggest that chlorosis tolerance could be ascribed to increased FCR (enhanced Fe uptake capacity) and POD enzyme activities at the leaf level. The absence of a similar adaptive strategy in inefficient lines may explain their susceptibility to Fe deficiency growth condition. Leaves of Fe-efficient potato plant lines maintained photosynthesis and had higher chlorophyll contents under IDC-promoting conditions than inefficient lines. Based on the leaf responses to Fe deficiency, results of this study show that the photosynthetic and POD antioxidant systems of chlorosis-sensitive lines were negatively affected by Fe starvation.

The differential tolerance to Fe deficiency among the two categories of plant lines can be linked to differences in antioxidant (POD) responses. It is suggested that both the roots

and leaves of Fe-efficient lines have enhanced capacities to scavenge and/or detoxify ROS. It is also suggested that plant lines with low-moderate chlorosis scores thrive under Fe-deficiency stress through elevated POD activity which signifies an improved ability to limit ROS production under this nutritional constraint. This implies that under Fe-deficiency stress conditions, plant lines that are Fe-efficient possibly evolve adaptive mechanisms to sustain POD activity as antioxidant response in order to protect cells from ROS-induced oxidative damage. Fe-inefficient plant lines may be incapable of activating and/or sustaining the biosynthesis of POD in response to Fe shortage and thus, unable to thrive under such condition. It is implied that POD antioxidant activity is potentially an important contributor to Fe deficiency tolerance in *Solanum tuberosum* cv 'Iwa' and increased activity levels could be used as an indicator of IDC tolerance.

FCR is a low-Fe inducible enzyme which facilitates ferric reduction and uptake of Fe. This study confirms that FCR plays a role in Fe uptake in potato roots and leaves since FCR activity was stimulated in response to Fe deficiency and much more in Fe-efficient plant lines than inefficient lines. The capacity of the roots and leaves of chlorosis tolerant plants to induce increased FCR activity under limiting Fe conditions could be used as a screening technique for Fe efficiency.

Transcriptional profile of iron homeostasis-related genes varied among roots and leaves and between plant lines. The A1 chlorosis tolerant plant line exhibited Fe-efficiency responses in leaf and root organs at the molecular level (increased *fer3* and *irt1* transcripts). Significantly increased *fer3* transcript levels were detected in the leaves of a higher proportion (62%) of the putative Fe-efficient lines than in roots (37.5%). The findings suggest that Fe-efficiency may be linked to enhanced *fer3* expression which serves to facilitate Fe acquisition and/or storage capacity in Fe-efficient plants probably as adaptive mechanism to enable survival or tolerance under Fe-deficiency stress conditions. *IRT1* expression levels were greater in roots than in leaves by 20%. *IRT1* expression in the roots of 50% of the potential Fe-efficient plant lines (A1, B2, B9, E3) was enhanced in response to Fe deficiency. The expression of *fer3* and/or *irt1* was however, high in some IFN lines and control plants compared to other EF lines. Gene expression (mainly *irt1*) in control plant roots and leaves may have been high because these plants had been grown under Fe sufficiency and as such responded much more rapidly upon exposure to Fe deficiency stress condition compared to some regenerants (EF and IFN lines) derived from IDC tolerant calli cultured on Fe-deficient medium. In all, the results presented herein confirm that *fer3* and *irt1*

play essential roles in Fe storage and transport principally in the leaves and roots respectively.

The results of the current study showed that some of the putative EF (especially A1, B2, B9) plant lines have both the capacity to elicit improved morphological and biochemical characteristics in responses to Fe-deficiency stress as well as enhance the expression of Fe homeostasis-related genes (*fer3* and *irt1*). These characteristics are most likely responsible the ability of Fe-efficient plants to grow, under Fe limiting conditions. Fe efficiency appears to involve the ability of plants to grow under Fe deficiency conditions and to activate specific responses in order to facilitate Fe uptake. Perhaps, the low/no chlorotic symptoms, EF-related morphological and/or biochemical responses displayed by other selected IDC tolerant plant lines may have been due to transient physiological responses or adaptation to Fe-deficiency which may not have necessarily resulted in IDC tolerance-related alterations at the gene level.

7.4 Applications of the findings

Fe deficiency-induced chlorosis is a serious nutritional problem worldwide mostly in regions with calcareous soils. Hence, the improvement in Fe efficiency in plants by selection of stable variants is vital. To the knowledge of the author, the development of Fe-efficient lines in potato has not previously been carried out using *in vitro* selection methods. The *in vitro* selection protocols designed can be adapted to other plants and serve as an approach to dealing with the problem of Fe chlorosis. The morphological, biochemical and molecular properties used to characterise the cell lines in this study could be valuably employed in screening programs intended for the selection of Fe-efficient genotypes. It is expected that the techniques employed and the selected plant lines obtained would open up an entirely new range of novel plant materials useful for enhanced IDC tolerance.

The plant lines generated in this study can serve as starting germplasm for purpose of plant breeding, preservation, and for use in other research. These plant lines can be useful materials in breeding programs aiming at selecting IDC tolerant genotypes. This study can make a contribution to agriculture in the enhancement of crop productivity in calcareous soils. The Fe-efficient lines can be beneficial for growth on such marginalised soils where Fe solubility and availability is compromised. Enhancement of Fe uptake capacity can boost crop productivity and enable plant growth in geographical regions originally unfavourable for optimal crop yields due to Fe-deficiency (Cianzio et al., 2006). This research is beneficial in contributing to agricultural efforts to minimise economic losses arising from low crop

productivity on soils deficient in Fe supply. The isolation of iron-efficient somaclonal variant plants can play a role in strategies aiming to avoid excessive iron fertiliser application. Obtaining potato variants with efficient use of iron and enhanced iron content is a crucial issue for populations worldwide especially given the current widespread increase in potato production and consumption globally with the biggest boost occurring in the developing world (FAO, 2011).

7.5 Recommendations for future research

It is envisaged that the findings of this study achieved within a limited timeframe (3 years) will trigger further studies into *in vitro* selection schemes for the selection of Fe-efficient plant lines and additional characterisation of such lines. Future research avenues arising from the results presented in this study include;

Due to the lack of ample chlorosis tolerant calli and the loss of some callus materials owing to their low morphogenic potential for plant regeneration, it was not feasible to perform extensive biochemical and molecular assays on the selected Fe-efficient calli. Therefore, in future, it would be beneficial to evaluate the morphological, biochemical and molecular characteristics of chlorosis tolerant callus cells.

It would be helpful to gain more knowledge on subcellular localisation and distribution of iron in callus cultures using high resolution and highly sensitive detection techniques such as Transmission electron microscopy, Secondary ionisation mass spectrometry, Laser ablation inductively coupled plasma mass spectrometry (LA–ICP-MS), Synchrotron X-ray fluorescence microtomography, or Micro-particle induced X-ray emission. These techniques together with the Perls histochemical Fe staining method, can aid in creating models for how responses to Fe deficiency lead to alterations in Fe uptake and translocation in calli and plants.

The determination of Fe content in *Solanum tuberosum* cv ‘Iwa’ tubers is beyond the scope of this study. Therefore, further work is necessary to develop effective tubularisation schemes for tuber development from the potato plant lines. Such future work could evaluate Fe concentration and investigate the association or relationship between Fe contents in potato tubers and differential tolerance to chlorosis. It will be interesting to know if the selected Fe-efficient cell lines would produce tubers with enhanced Fe content.

The novel materials generated in this study are promising. Therefore, the *in vitro* techniques applied should be explored with other potato cultivars or plants. This will aid in validating the methods used and the results attained in this study.

In vitro selection in combination with traditional breeding techniques can yield valuable new genotypes. A limitation of *in vitro* culture is that it cannot simulate a field environment and plant response may be variable in field trials than in *in vitro* tests. Greenhouse and field evaluation will be necessary in future to validate the Fe-efficiency trait in the plant lines obtained in this work. Further work is needed to investigate whether the Fe-efficiency trait is stable under low-Fe availability conditions especially in calcareous soils and find out if the plant lines exhibit characteristics associated with IDC tolerance.

Since iron deficiency can influence the accumulation of other metals such as Mn, Zn and Cu (Legay et al., 2012), research opportunities exist for investigations into the morphological and biochemical responses of potato callus cultures to the deficiency of the micronutrients, Zn and Mn, which can be co-transported with Fe by IRT1 (Korshunova et al., 1999; Eide et al., 199).

Gonza´lez-Vallejo et al. (2000) argued that FCR activities of whole leaf pieces may include other reducing activities such as the leakage of reducing organic anions at the leaf wound. These authors indicate that such activities appear not to be related to FCR activity of leaf plasma-membrane. In future it will be interesting to do follow-up work designed to evaluate plasma-membrane bound FCR activity using leaf mesophyll cells from the INF and EF plant lines established here.

Iron deficiency can cause increases in organic acid metabolism and concentrations in roots, leaves and exudates of plants (Jelali et al., 2013; Abadía et al., 2002). Such responses are related to better Fe-use efficiency and give an indication of the Fe chlorosis tolerance level of a plant (Jelali et al., 2013). Only phenolic content was determined in the present study. Hence, it would be helpful to gain more knowledge relating to the impact of Fe-deficiency on organic acids (e.g. citrate, malate, and amino acids) contents at the callus and plant levels. An important question for future studies using the plant lines generated in this study is to determine the correlation between Fe deficiency and organic acids accumulation.

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APPENDICES

Appendix A: Culture media

A1. Murashige and Skoog (MS) tissue culture medium (1962)

Table A1. The amounts of major, minor and organic salts required for 500 ml stock solutions

Major salts (10X stock)	
NH_4NO_3	8.25 g
KNO_3	9.5 g
$CaCl_2.2H_2O$	2.2 g
$MgSO_4.7H_2O$	1.85 g
KH_2PO_4	0.85 g
Minor salts (100X stock)	
KI	0.0415 g
H_3BO_3	0.310 g
$MnSO_4.4H_2O$	1.115 g
$ZnSO_4.7H_2O$	0.43 g
$CuSO_4.5H_2O$	0.00125 g
$CoCl_2.6H_2O$	0.00125g
$Na_2MoO_4.2H_2O$	0.0125 g
Organic Supplement (100X stock)	
Myo-inositol	5 g
Nicotinic acid	0.025 g
Pyridoxine-HCl	0.025 g
Thiamine-HCl	0.005 g
Glycine	0.1 g
Iron stock (100X stock)	
A: $FeSO_4.7H_2O$	1.39 g in 200 ml of distilled water
B: $Na_2EDTA.2H_2O$	1.865 g in 200 ml of distilled water

Major, minor and organic salts stock solutions were brought to volume (500 ml) with distilled water. For iron stock solution, solutions A and B were mixed together and the volume was adjusted to 500 ml with distilled water and kept in a brown bottle. All solutions were stored at 4^{o}C.

Table A2. Volumes of the stock solutions and water required for preparing different volumes (indicated in the first row) of half-strength MS medium

	250ml	500ml	1000ml
Major salts	12.5	25	50
Minor salts	1.25	2.5	5
Organic salts	1.25	2.5	5
Iron stock	1.25	2.5	5
Sucrose(30%w/v)	7.5	15	30
Distilled water	220	400	800

Steps involved in the preparation of MS medium for use in basic potato tissue culture

1) Stock solutions, sucrose and distilled water were mixed as indicated in (II) and the solution stirred until sucrose dissolved

2) pH of medium adjusted to 5.7-5.8*

3) Dispense the media**

4) Addition of agar (0.8% w/v)

5) Autoclave media.

*where necessary, the required amounts of plant growth regulators were added before pH adjustment to 5.7-5.8.

** media were dispensed into glass bottles and autoclaved after which they were poured into pre-sterilised petri dishes or pre-autoclaved tissue culture vessels.

A2 Different medium formulations for callus induction

Table A3. Concentrations (µM) of NAA and BA supplemented into half-strength MS medium

	Plant growth regulators	
Growth medium	BAP (µM)	NAA (µM)
Control (½ MS)	0	0
N1	0	1.07
N3	0	3.22
N5	0	5.37
N7	0	10.7
B1	0.89	0
B3	2.66	0
B5	4.44	0
B7	8.88	0
NB1	0.89	1.07
NB2	0.89	3.22
NB3	0.89	4.30
NB4	0.89	5.37
NB7	0.89	10.7
NB8	1.78	1.07
NB9	1.78	2.15
NB10	1.78	3.22
NB11	1.78	4.30
NB14	1.78	10.7
NB15	2.66	1.07
NB16	2.66	2.15
NB17	2.66	3.22
NB18	2.66	4.30
NB22	3.55	1.07
NB23	3.55	2.15
NB24	3.55	3.22
NB25	3.55	4.30
NB27	3.55	8.05
NB33	4.44	5.37
NB34	4.44	8.05
NB35	4.44	10.7
NB42	6.66	10.7
NB43	8.88	1.07
NB49	8.88	10.7

Table A4. Iron ($FeSO_4.7H_2O$) stock solutions of different concentrations.

[µM] ½MS	P (Stock)	P (MS) in mg/l	P (½MS) in mg/l
0.001	0.03 mg/500ml	5.56E-04	2.78E-04
0.005	0.14 mg/500ml	2.78E-03	1.39E-03
0.01	0.28 mg/500ml	5.56E-03	2.78E-03
0.05	1.39 mg/500ml	2.78E-02	1.39E-02
0.1	2.78 mg/500ml	5.56E-02	2.78E-02
0.5	13.9 mg/500ml	0.28	0.14
1	27.8 mg/500ml	0.56	0.28
5	139 mg/500ml	2.78	1.39
10	278 mg/500ml	5.56	2.78
20	556 mg/500ml	11.1	5.56
30	834 mg/500ml	16.7	8.34
40	1.11 g/500ml	22.2	11.1
50	1.39 g/500ml	27.8	13.9
60	1.67 g/500ml	33.4	16.7
70	1.95 g/500ml	38.9	19.5
80	2.22 g/500ml	45.0	22.2
90	2.50 g/500ml	50.0	25
100	2.78 g/500ml	55.6	27.8
200	5.56 g/500ml	111	55.6
400	11.1 g/500ml	222	111
800	22.2 g/500ml	445	222

P: mass concentration; ½MS: half-strength MS

$Na_2EDTA.2H_2O$ (50 µM): 1.86 g in 500 ml distilled water

Preparation of half-strength MS medium of different iron concentrations.

a) For FeNaEDTA stock solution, the required amount of $FeSO_4.7H_2O$ was weighed into 200 ml of MilliQ water and mixed with $Na_2EDTA.2H_2O$ solution (1.86 g $Na_2EDTA.2H_2O$ in 200 ml distilled water).
b) The mixture was stirred and the volume adjusted to 500 ml with distilled water. The iron stock solutions were kept at 4^oC and away from light (brown bottle or wrapped in aluminium foil) until used.
c) A volume of 2.5 ml of FeNaEDTA stock solution was added to 497.5 ml of half-strength MS to make up 500 ml giving the corresponding FeNaEDTA concentrations (0.001-800 µM) used for experiments.

Appendix B: Biochemical assays

B1. Stock solutions for FCR enzyme activity

100 ml of 50 mM MES at 195.2 $gmol^{-1}$ = 0.976 g

100 ml of 1 mM Fe(III)-EDTA at 367.06 g/mol = 36.71 mg (freshly prepared)

50 ml of 0.5 mM BPDS at 536.50 g/mol = 13.41 mg (freshly prepared)

A 50 ml FCR assay medium (freshly prepared), was made up as follows

5 ml of 50 mM MES buffer (5 mM working solution)

5 ml of 1 mM Fe(III)-EDTA (0.1 mM working solution)

30 ml of 0.5 mM BPDS (0.3 mM)

10 ml of half-strength MS medium without iron supplement

B2. Solutions for total phenolics assay

Folin and Ciocalte Fu's Phenol Reagent (1/10): 1 ml reagent added to 9 ml distilled water (equiv. 0.2M, freshly diluted)

80% (v/v) acetone: 20 ml of distilled water mixed with 80 ml acetone

7.5% (w/v) Na_2CO_3: 7.5 g of Na_2CO_3 in 100 ml distilled water

Fresh solution of gallic acid (1 mg/ml) was made up in 80%acetone

Table B1. Gallic acid concentrations

Gallic Acid per Assay (µg)	Volume of gallic acid stock (µl)	Volume of H_2O (µl)
0	0	500
4	20	496
8	40	492
12	60	488
16	80	484
20	20	480

B3. Lipid peroxidase (TBARS) assay

0.1 % (w/v) trichloroacetic acid: 0.1 g in 100 ml distilled water

20% (w/v) trichloroacetic acid (TCA) : 20 g in 100 ml distilled water

0.5% (w/v) thiobarbituric acid: 0.5 g in 50 ml of 20% TCA and brought to 100ml volume with distilled water

B4. Preparation of 3, 3-diaminobenzidine (DAB) staining solution

- Added 0.1 N HCl (1-2 drops) to 50 ml distilled water till pH was 3.6.
- Weighed 10 mg of DAB into 10 ml of above solution into tubes to make up 1 mg/ml.
- Tubes were closed, wrapped with aluminum foil and vigorously shaken at 37 °C in an orbital shaker for at least 30 min

Completely dissolved DAB solution was colourless or slight pink (used fresh)

B5. Preparation of phosphate buffers

Preparation of sodium phosphate buffers according to Sambrook et al. (2001)

1) Aliquots of 1M stock solutions were pipetted into a volumetric flask according to the desired pH of buffer (see table B2 below).

2) Water was added and the solution stirred

3) pH of solution was measured. 1M NaOH was added to raise pH if below the desired pH and 1M HCl to lower pH if it is above the desired

4) Water was carefully added to make up the total volume desired

- 1M stock buffer solutions

100 ml of 1M Na_2HPO_4 at 142 g/mol = 14.20 g

100 ml of 1M NaH_2PO_4 at 156.01 g/mol = 15.60

500 ml of 1M K_2HPO_4 at 174.18 g/mol = 87.09g

500 ml of 1M KH_2PO_4 at 136.09 g/mol = 68.05g

Table B2 Volumes of weak acid and base for buffer preparation

(i)

50 ml of 0.1M Sodium Phosphate Buffer (25°C)		
pH	Volume of 1 M Na_2HPO_4 (ml)	Volume of 1 M NaH_2PO_4 (ml)
6.0	0.6	4.4
6.2	0.89	4.11
6.4	1.28	3.73
6.6	1.76	3.24
6.8	2.32	2.69
7.0	2.89	2.12
7.2	3.42	1.58
7.4	3.87	1.13
7.6	4.23	1.78

(ii)

1 Liter of 0.05 M Phosphate Buffer (25°C)		
pH	1 M Monobasic Solution (ml)	1 M Dibasic Solution (ml)
6.6	32	18
6.7	29.8	20.2
6.8	26.5	23.5
6.9	24	26
7	21.1	28.9
7.1	18.4	31.6
7.2	16.8	34.2
7.3	13.4	36.6
7.4	11.2	38.8
7.5	9.4	40.6
7.6	7.8	42.2

iii)

1 Litre of 0.1M Potassium Phosphate Buffer (25°C)		
pH	Volume of 1 M K_2HPO_4 (ml)	Volume of 1 M KH_2PO_4 (ml)
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4

B6. Reagents and solutions for Perls stain

Concentrated HCl is 11M and 36.5% pure

11 M x 36.5% = 4015%

1L = 40.15g

- To prepare 100 ml of 4% (v/v) HCl,

About 80 ml of distilled water was measured into 100 ml volumetric flask and 10 ml of 11 M HCl was pipetted into the water. Solution was stirred and topped up with water to the 100 ml mark

- 4% (w/v) Potassium ferrocyanide

Weighed 4g of potassium ferrocyanide into about 50 ml distilled water

Solution stirred until complete dissolution and distilled water was added to a 100 ml mark

- Methanolic solution (0.01M NaN_3 and 0.3% H_2O_2)

200 ml of 0.01M NaN_3 at 65.01 $gmol^{-1}$ = 0.13 g

0.3% (v/v) of H_2O_2: 1 ml of 30% (v/v) H_2O_2 in 100 ml diluent

To 10 ml of methanol, 0.13 g of NaN_3 was added and mixed until completely dissolved

2 ml of 30% (v/v) H_2O_2 was added and mixed by stirring

Topped up to the 200 ml mark with distilled water

- Fixation solution

2% w/v parafolmaldehyde: 2 g in 100 ml extraction buffer

1% w/v caffeine: 1 g in 100 ml buffer

1% v/v glutaraldehyde: 4 ml of 25% glutaraldehyde in 100 ml buffer

To 50 ml of 0.1M sodium phosphate buffer (pH=7.0), 2 g of parafolmaldehyde, 1 g caffeine and 4 ml of glutaraldehyde were added

Solution was stirred to completely dissolve components and brought to 100 ml with buffer.

B7. Antioxidant enzyme extraction buffer (total volume: 100 ml)

- 50 mM phosphate buffer (pH 7.0)
- 1% (w/v) polyvinylpolypyrrolidone (PVP-40): 1g PVPP in 100 ml extraction buffer
- 1 mM phenylmethylsulfonylfluoride (PMSF)
- 5 mM ascorbate (added only for APX enzyme extracts)

- PMSF stock solution (10 mM; 50 ml)

50 ml of 10 mM PMSF at 174.19 $gmol^{-1}$ = 87.10 mg

Weighed 87.10 mg of PMSF, dissolved in isopropanol and topped up to 50 ml.

Pippeted 10 ml of 10 mM PMSF for a 100 ml extraction buffer volume.

- Ascorbate stock solution (50 mM; 100 ml)

100 ml of 50 mM at 176.13 g/mol = 0.88g

Added 10 ml of 50 mM ascorbate to make up the 5 mM required for a 100 ml extraction buffer.

For a 1 ml assay reaction mixture, 10 µl of 50 mM ascorbate was used.

Dissolved PVP (1 g) and PMSF (10 ml) in 80 ml of 50 mM phosphate buffer (pH 7.0) and completed the volume to 100 ml with the buffer. Ascorbate was added in the case of APX extraction buffer.

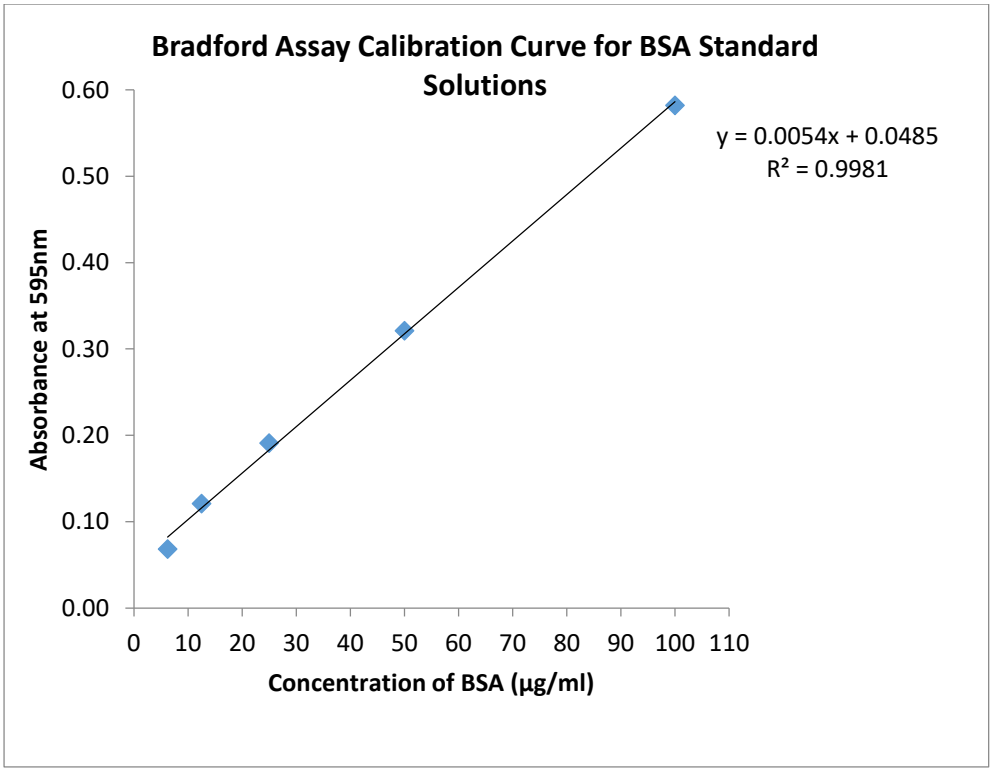

Figure B1. Calibration curve for BSA standard solutions used in Bradford assay

Table B3. Varimax rotation factor scores for the three factor PCA model for callus cultures exposed to 0-5 and 50µM Fe medium for one month

Parameters	PC1	PC2	PC3	Communalities
APX activity	0.29	**0.53**	0.52	0.63
POD activity	**0.76**	0.09	0.19	0.63
CAT activity	-0.03	**0.93**	0.08	0.87
FCR activity	-0.72	0.03	**0.05**	0.52
Chlorophyll	0.75	-0.36	**0.25**	0.75
Carotenoids	-0.01	0.07	**0.84**	0.72
Phenolics	-0.08	0.82	-0.16	0.70
TBARS levels	-0.76	-0.07	-0.13	0.60
Fresh weight	0.28	-0.23	**0.71**	0.64
Eigenvalue	2.88	2.01	1.16	
Percentage total varaince	32.02	22.38	12.87	
Cummulative percentage total variance	32.02	54.4	67.27	

Table B4. Varimax rotation factor scores for the three factor PCA model for callus cultures exposed to 0-5 and 50µM Fe medium for three months

Parameters	PC1	PC2	Communalities
APX activity	**0.60**	0.51	0.62
POD activity	**0.85**	-0.22	0.77
CAT activity	**0.83**	0.07	0.69
FCR activity	-0.18	**0.46**	0.25
Chlorophyll	**0.88**	-0.23	0.83
Carotenoids	**0.78**	-0.28	0.68
Phenolics	-0.02	**0.82**	0.68
TBARS levels	**0.76**	0.05	0.58
Fresh weight	**0.89**	-0.08	0.81
Eigenvalue	4.6	1.31	
Percentage total varaince	51.13	14.50	
Cummulative percentage total variance	51.13	65.62	

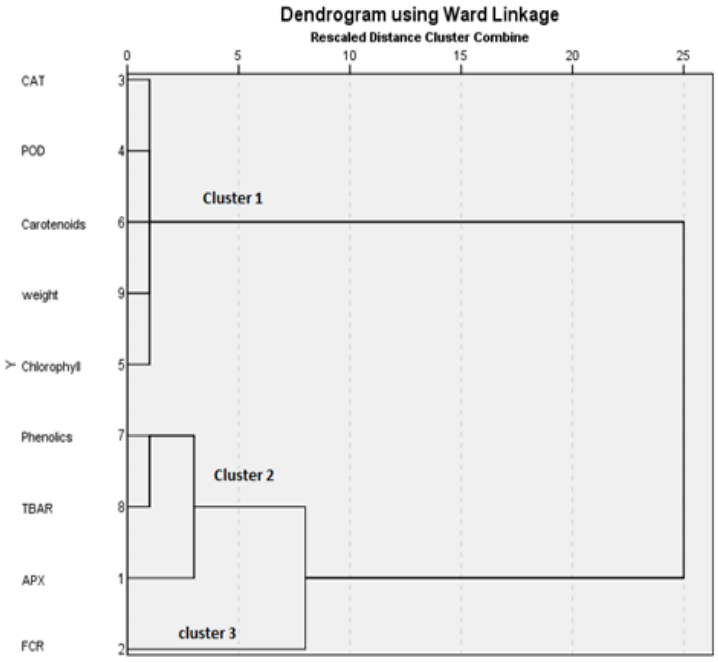

Figure B2. A dendrogram showing clustering of morphology and biochemical responses of potato calli exposed to iron deficiency treatments for a month.

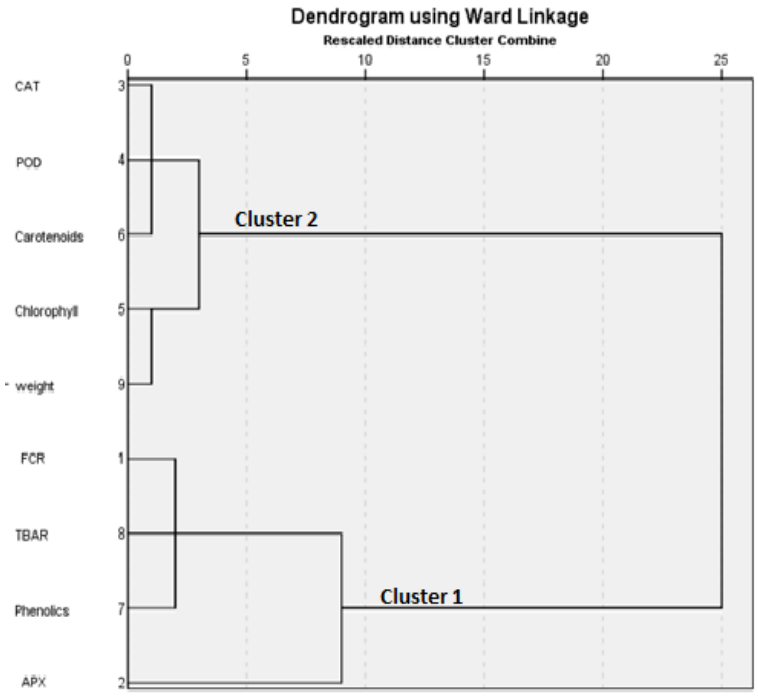

Figure B3. A dendrogram showing clustering of morphology and biochemical responses of potato calli exposed to iron deficiency treatments for three months.

Table B5. Varimax rotation factor scores for the two factor PCA model for leaves of potato plant lines. Highest factor loadings for components are bold faced and underlined. Potential selected Fe-efficient lines are italicised.

Cell lines	PC1	PC2	Communalities
A1	**0.75**	0.66	1.00
A2	**0.75**	0.66	1.00
A3	0.69	**0.73**	0.99
A4	0.65	**0.76**	0.99
B1	0.61	**0.79**	0.99
B2	**0.72**	0.69	1.00
B3	0.69	**0.72**	0.99
B4	0.6	**0.8**	0.99
B5	**0.73**	0.68	0.99
B8	0.7	**0.71**	0.99
B9	**0.74**	0.67	0.99
B10	**0.76**	0.65	1.00
B12	0.53	**0.85**	0.99
B16	0.65	**0.76**	0.99
C1	**0.78**	0.62	0.99
C2	**0.8**	0.6	0.99
C3	**0.78**	0.62	0.99
C4	**0.8**	0.6	0.99
C5	0.67	**0.73**	0.98
D1	**0.8**	0.61	0.99
D2	**0.81**	0.58	0.99
D3	**0.81**	0.59	1.00
D4	**0.74**	0.67	0.99
E1	**0.79**	0.61	1.00
E2	**0.77**	0.63	0.99
E3	**0.79**	0.61	1.00
E4	**0.8**	0.6	1.00
E5	**0.79**	0.61	1.00
E6	**0.79**	0.61	0.99
E7	**0.79**	0.61	0.99
E8	**0.78**	0.62	1.00
E9	**0.79**	0.61	0.99
E12	**0.79**	0.62	0.99
E15	**0.78**	0.62	0.99
Eigenvalue	33.62	0.31	
Percentage total varaince	98.88	0.91	
Cummulative percentage total variance	98.88	99.79	

Table B6. Varimax rotation factor scores for the two factor PCA model for potato plant lines. Highest factor loadings for components are bold faced and underlined. Potential Fe-efficient lines are italicised.

Cell lines	PC1	PC2	Communalities
A1	**0.97**	0.25	1.00
A2	**0.93**	0.34	0.98
A3	0.64	**0.76**	1.00
A4	**0.80**	0.60	1.00
B1	**0.90**	0.43	0.99
B2	**0.97**	0.24	0.99
B3	**0.81**	0.57	0.99
B4	0.66	**0.74**	0.99
B5	**0.92**	0.34	0.95
B8	**0.76**	0.64	0.99
B9	**0.96**	0.25	0.99
B10	**0.95**	0.31	1.00
B12	0.63	**0.77**	1.00
B16	**0.70**	**0.70**	0.99
C1	0.50	**0.86**	1.00
C2	0.36	**0.93**	0.99
C3	0.51	**0.86**	1.00
C4	0.20	**0.98**	0.99
C5	0.04	**1.00**	1.00
D1	**0.75**	0.65	0.99
D2	**0.78**	0.60	0.97
D3	**0.81**	0.58	0.98
D4	**0.78**	0.57	0.94
E1	**0.85**	0.53	0.99
E2	**0.84**	0.54	0.99
E3	**0.83**	0.55	1.00
E4	0.63	**0.77**	0.99
E5	0.47	**0.88**	0.99
E6	**0.77**	0.63	1.00
E7	**0.75**	0.65	0.99
E8	0.68	**0.72**	0.98
E9	**0.84**	0.54	1.00
E12	0.64	**0.76**	0.99
E15	0.60	**0.79**	0.99
Eigenvalue	30.8	2.822	
Percentage total varaince	90.59	8.3	
Cummulative percentage total variance	90.59	98.886	

Appendix C. Nucleic acids analysis

C1. Agarose gel electrophoresis

- 25 x TAE Buffer stock

121 g Tris-base in water

28.5 ml Glacial acetic acid

9.3 g Na2 EDTA

Adjust pH = 7.6 and adjust volume to 1 L with distilled H_2O

A working solution of 1x TAE buffer was prepared in a IL volumetric flask. A 25x TAE buffer stock of 40 ml was diluted with 960 ml MilliQ water.

- Gel loading buffer (6 x agarose gel loading buffer)

60% Glycerol

60 mM EDTA

10 mM Tris-HCl (pH 7.6)

0.03% xylene cyanol

0.03% bromophenol blue

SYBR Safe

C2. 1 M Tris Stock

121 g Tris base

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCl